Molecular Mechanisms Regulating Pluripotency in Rat and Mouse Embryonic Stem Cells

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"Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning."

— Albert Einstein, Relativity: The Special and the General Theory

Abstract

Pluripotency is defined as the capacity at the single cell level to initiate the formation of all lineages of the mature organism in response to the signals from the embryo or cell culture environment. Studying pluripotency is the foundation of mammalian development and embryonic stem cell (ESC) biology. However, how pluripotency can be captured and maintained still remains an intriguing question with many practical implications.

ESCs can be derived and propagated from both mouse and rat strains. The rat has long been an essential model of human health and disease and is traditionally preferred over mice in many areas of the biomedical research. However, mouse models have gained popularity over rats as a preferred model due to the inability to genetically manipulate the rat genome in the sophisticated manner as in the mouse. Derivation of germline-competent rat ESCs from a wide variety of rat strains will pave the way to targeted genetic manipulation of the rat genome. Nevertheless, rat ESC culture system is still in its earlier stages and deeper understanding of molecular mechanisms regulating pluripotency in rat ESCs is essential.

We devised to establish authentic Brown Norway rat ESCs. Brown Norway rats are the most widely used strain in the biomedical research and have an edge over the other strains due to the decoding of its genome sequence. In the rat project, Brown Norway ESCs were derived using defined culture conditions and characterized for their pluripotency. The established ESCs expressed the characteristic ESC markers and were able to induce teratoma formation. Due to technical difficulties and host incompatibility issues, it was nevertheless not possible to generate germline competent ESCs. Stabilization of pluripotency in ESCs is a key question in order to efficiently derive and cultivate these cells. Recently, a new gene family (Preferentially expressed antigen in melanoma-like, Pramel) was identified, which might play an important role in maintenance of pluripotency in mouse and rat ESCs. In a parallel project on mouse ESCs (mESCs), we aimed at understanding the molecular factors regulated by Pramel7, a member of this family. Pramel7 is a novel factor involved in maintenance of pluripotency in mESCs, which is regulated through LIF/STAT3 pathway. LIF regulates transcription of *Pramel7* via STAT3. *In vivo*, Pramel7 is expressed in the pluripotent cells of the morula and blastocyst. Knockdown of *Pramel7* induces differentiation of mESCs whereas the overexpression maintains them pluripotent even in the absence of LIF. Transcription of *Pramel7* is reported to be blocked in the presence of GSK3β inhibitor. GSK3β is an important player in three different signalling pathways, PI3K/Akt, Shp2/MAPK and Wnt/ β-catenin pathway, all of which are known to play key roles in the self-renewal and differentiation of pluripotent ESCs.

To deeper understand the link between GSK3β /β-catenin and Pramel7 in ESCs, genetic and biochemical examination of ESCs cultured in defined conditions was carried out. Analysis of intracellular signalling pathways along with the genetic mutants of the target genes, β-catenin and GSK3β sheds light on the circuitry involved. Initially, the question whether there is a link between β-catenin and Pramel7 was addressed. We observed

that *Pramel7* was upregulated in β-catenin KO ESCs. Signalling mutants of β-catenin were derived and further experiments on these mutants did not indicate a direct involvement of β-catenin in *Pramel7* transcription. In the next step, effect of various GSK3β mutations on Pramel7 transcription was analysed. It was observed that knockdown of *GSK3β* using siRNA technology resulted in abrogation of *Pramel7* transcription. However, kinase function of GSK3β alone did not modulate Pramel7 expression. Further experiments revealed that blocking activation of ERK, downstream of FGF4, in presence of GSK3β upregulated *Pramel7*. We propose that derepression of GSK3β along with the inhibition of ERK leads to the repression of a transcriptional repressor of Pramel7.

Zusammenfassung

Der Begriff der Pluripotenz wird definiert als die Fähigkeit einer Zelle, auf ein Signal von Embryo oder Zellkulturumgebung hin die Formation aller drei Keimblätter eines reifen Organismus zu bilden. Die Erforschung der Pluripotenz stellt somit eine Basis in der Biologie der Säugetierentwicklung, aber auch in der embryonalen Stammzellforschung dar. Trotzdem sind Fragen, etwa wie der Zustand der Pluripotenz stabilisiert und beibehalten werden kann, immer noch nicht vollumfänglich geklärt und bergen auch viele praktische Herausforderungen.

Embryonale Stammzellen (ESCs) können von den beiden Modellorganismen Maus und Ratte erzeugt und erhalten werden. Dabei ist die Ratte schon lange als Modell zur Erforschung humaner Erkrankungen bekannt und wird der Maus traditionellerweise in vielen Bereichen der biomedizinischen Forschung vorgezogen. Dennoch hat die Popularität von Mausmodellen in den letzten Jahren stark zugenommen. Der Hauptgrund dafür liegt darin, dass im Bereich ausgereifter genetischer Manipulationen das Rattengenom demjenigen der Maus weit hinterherhinkt. Um diese Lücke zu schliessen müssen keimbahnkompetente ESCs der Ratte (rESCs) von vielen verschiedenen Stämmen erzeugt werden. Dies erlaubt in einem nächsten Schritt eine gezielte genetische Mutagenese des Rattengenoms. Da aber auch die Kultivierung von rESCs immer noch in ihren Anfängen steht, ist es unumgänglich, Verständnis und Wissen über die Erhaltung der Pluripotenz von rESCs zu erweitern. In der biomedizinischen Forschung werden grundsätzlich Ratten des *Brown Norway*-Stamms verwendet, da deren Genom schon komplett sequenziert und decodiert worden ist. In diesem Projekt wurde nun geplant, authentische rESCs dieses Stamms zu etablieren. In einem ersten Schritt wurden dazu *Brown Norway*-rESCs unter definierten Kulturbedingen erzeugt und anschliessend auf deren Pluripotenz getestet und charakterisiert. Die rESCs exprimierten die charakteristischen Pluripotenzmarker und zeigten die Fähigkeit, nach Injektion in immunsupprimierte Mäuse, Teratomas auszubilden. Wegen technischer Schwierigkeiten und Inkompatibilität der Stämme von rESCs und Ammenmutter war es jedoch nicht möglich, keimbahnkompetente rESCs zu generieren.

Eines der grössten Hindernisse zur effizienten Erzeugung und Kultivierung von ESCs ist die Stabilisierung ihrer Pluripotenz. Neulich wurde die neue Familie der *Pramel*-Gene (Preferentially expressed antigen in melanoma-like) identifiziert, deren Mitglieder eine wichtige Rolle in der Pluripotenzerhaltung von ESCs von Maus und der Ratte spielen könnten. Deshalb zielten wir in einem Parallelprojekt darauf ab, die molekularen Faktoren zu identifizieren, welche von Pramel7, einem Mitglied der Pramel-Familie, reguliert werden. Pramel7 wurde vor Kurzem als ein in die Pluripotenzerhaltung von murinen ESCs (mESCs) involvierter Faktor identifiziert, welcher durch den LIF/STAT3-Signalweg gesteuert wird, wobei LIF (Leukemia inhibitory factor) die Transkription von *Pramel7* via STAT3 reguliert. *In vivo* ist Pramel7 in pluripotenten Zellen von Morula und Blastozyste exprimiert. *In vitro* induziert ein Knockdown von Pramel7 die Differenzierung von mESCs, während dessen Überexpression die Zellen in einem pluripotenten Zustand zu halten vermag, auch in Absenz von LIF. Zudem wurde gezeigt,

dass die Anwesenheit eines Inhibitors für GSK3β die Transkription von *Pramel7* inhibiert. GSK3β seinerseits hat wichtige Rollen in drei verschiedenen Signalwegen, nämlich in der PI3K/Akt-, der Shp2/MAPK- und der Wnt/β-catenin-Kaskade. Alle drei Signalwege werden unter anderem auch mit Selbsterneuerung und Differenzierung von pluripotenten mESCs in Verbindung gebracht.
Um etwaige Zusammenhänge zwischen GSK3β/β-catenin und Pramel7 zu erörtern, wurden mESCs, kultiviert unter definierten Bedingungen, genetisch und biochemisch untersucht. Eine Analyse der intrazellulären Signalwege mit Hilfe genetischer Mutanten der beiden Zielgene *β-catenin* und *GSK3β* sollte dabei helfen, mögliche Interaktionen und Zusammenhänge ans Licht zu bringen. Anfänglich wurde die Frage gestellt, ob eine Verbindung zwischen β-catenin und Pramel7 existiert, da in β-catenin-KO mESCs eine Hochregulierung von *Pramel7* festgestellt werden konnte. Für weitere Untersuchungen wurden als nächstes Signalmutanten von β-catenin erzeugt; weitere Experimente mit ihnen zeigten aber keinen direkten Einfluss von β-catenin auf die Transkription von *Pramel7*. In einem nächsten Schritt wurde getestet, ob verschiedene Mutationen von GSK3β einen Effekt auf die transkriptionelle Aktivität von *Pramel7* zur Folge haben. Hier kam zum Vorschein dass ein Knockdown von GSK3β durch siRNA die Aktivität von *Pramel7* zum Erliegen brachte. Es ist aber zu erwähnen, dass die GSK3β-Kinasefunktion allein die Expression von *Pramel7* nicht zu ändern vermochte. Aus weiteren Experimenten konnte dann gezeigt werden, dass in Anwesenheit von GSK3β eine Blockierung von ERK, downstream von FGF4, *Pramel7* hochregulierte. Daraus schliessen wir, dass eine Derepression von GSK3β einhergehend mit einer Inhibierung von ERK zu einer Unterdrückung eines transkriptionellen Repressors von *Pramel7* führt.

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We don’t remember days, we remember moments. CESARE PAVESE ☺

Table of contents

List of figures

List of tables

Abbreviations

2i	2 inhibitors	Klf4	Kruppel-like family 4
β-cat	β-catenin	LY	LY294002
Bmp4	bone morphogenetic protein 4	LIF	Leukemia inhibitory factor
CH	CHIR99021	PGC	primordial germ cell
ERK	extracellular receptor kinase	PI3K	phosphatidylinositol 3-kinase
ESR1	Estrogen receptor 1	PKB	protein kinase B
EpiSCs	Epiblast stem cells	PBS	Phosphate buffered saline
EBs	embryoid bodies	PD	PD0325901
ESCs	Embryonic stem cells	Pramel7	Preferentially expressed antigen in melanoma like 7
FGF	fibroblast growth factor	QRT-PCR	Quantitative real time PCR
FGFR	FGF receptor	RT	Room temperature
GSK3β	Glycogen synthase kinase 3 beta	SH2	src homology 2
ICM	inner cell mass	SOCS	suppressor of cytokine signalling
IL-6	interleukin 6	SSC	spermatogonial stem cell
iPS	induced pluripotent stem	STAT3	signal transducer and activator of transcription 3
IP	Immunoprecipitation	S727	Serine 727 residue
JAK	janus activated kinase	WT	Wild type
KO	knockout	Y705	Tyrosine 705 residue

Outline

The thesis mainly comprises of two projects. Below is an outline of the framework of the thesis.

First part of the introduction begins with a generalised summary of stem cells and their applications followed by defining the term “pluripotency”. The next two chapters focus on the importance of rat in biomedical research and describe the different gene targeting methodologies developed over the past years. The last part of the introduction gives a broad overview on the embryo-derived stem cells from the mouse, the intrinsic factors and the different extracellular signaling pathways involved in the maintenance of pluripotency. This is followed by the detailed description about Pramel7, the gene of interest for this project. The introduction is concluded by stating the goal of the thesis.

The results section is split into two parts representing the two projects. In the first part, the results for the rat project are described. Regarding the rat project, establishment and characterization of the rat embryonic stem cells is presented. The second part of the results section describes the different molecular mechanisms involved in regulating the expression of Pramel7 at the transcriptional level.

The initial paper, which created the groundwork for the second project has been listed at the end of the thesis as Annex.

The discussion chapter follows the structure of the results sections and is divided into two parts. In regard to the first project, the technical and the scientific issues faced during the establishment and characterization of rat embryonic stem cells are discussed. Concerning the second project, the findings relevant to the mechanisms controlling Pramel7 transcription via different pathways involving GSK3β are reviewed and a hypothetical model representing the regulation of Pramel7 transcription has been demonstrated.

Finally, on the basis of the findings in the thesis, general conclusions are drawn and different experimental approaches are proposed that can be used to develop a clearer understanding of molecular mechanisms regulating pluripotency in both rat and mouse embryonic stem cells.

A. Introduction

1. Stem cells and their applications

Stem cells are specialized cells present in the multicellular organisms, which replace the existing injured, damaged or terminally differentiated cells thus maintaining tissue homeostasis. They are defined by their ability to self-renew and undergo differentiation. Self-renewal is a property of the cell to divide into two daughter cells where either one or both daughter cells are identical to the pre-existing one i.e. one remains a stem cell and other may differentiate into a specialized cell type. Differentiation, on the other hand, means the transition of a stem cell into a more specialized cell type losing the ability to multiply itself and thus self-renew (Smith, 2001). They can be classified into totipotent, pluripotent, multipotent and unipotent based on their developmental potential as shown in Table 1.

Table 1: Definition of developmental options accessible to the cells

Totipotent	Form all the tissues of the organism including extraembryonic tissue	Zygote and early blastomeres of the mammalian embryo
Pluripotent	Can differentiate into all 3 germ layers of an organism	Embryonic stem cells
Multipotent	Can differentiate into all cell types of a specific lineage	Adult stem cells like haematopoietic stem cells
Unipotent	Can differentiate into cells of one type	Spermatogonial stem cells

These properties make them a very important tool for not only understanding the developmental processes but also developing stem cell based therapies for regenerative medicine. These attributes expand the horizon of studying mammalian embryogenesis and early differentiation processes *in vitro*, allow gene manipulation to study the role of specific genes, make them an important player in pharmaceutical field for drug development and toxicological testing. Most importantly, indefinitely renewable supply of cells makes them a possible source to treat huge spectrum of human ailments spanning neurodegenerative disorders (Brüstle et al., 1999), diabetes (Melton, 2011) and myocardial infarction (Miyahara et al., 2006). However, it is a long way off before this becomes a reality. There are many potholes that need to be filled in order to reach this goal. A major problem lies in the tumor formation from undifferentiated or inefficiently differentiated stem cells after expansion *in vitro*, which may be overcome by

applying appropriate methods to eliminate any such cells. Another question that still needs to be addressed is whether these stem cell derived differentiated cell types are fully functional after the transplantation, which prompts us towards establishing better models for transplantation. The biggest hurdle in cell replacement therapy still remains the immunological rejection, which may be obviated through reprogramming adult cells obtained directly from the patient (Takahashi and Yamanaka, 2006). However, the prerequisite for overcoming these problems lies in the thorough and deeper understanding of molecular mechanisms regulating pluripotency of stem cells.

Stem cells are broadly categorized into adult, embryo-derived and induced pluripotent stem cells, which will be discussed later. Phenomenon of pluripotency and self-renewal is discussed below.

2. Pluripotency and Self-renewal

Pluripotency is defined as the ability of a cell to give rise to all cell types of an embryo and adult. It is a property that is possessed by ESCs. The pluripotency of ESCs can be determined through both *in vivo* and *in vitro* differentiation. *In vitro* differentiation can be achieved by formation of embryoid bodies (EBs) from the ESCs. They are formed when ESCs are grown either by drops hanging from the lid or by culturing them on non-adherent dishes. ESCs then aggregate and differentiate into derivatives of all three germ layers (Martin and Evans, 1975). Another test is through *in vivo* differentiation in which ESCs are either injected in the kidney capsules, testis or subcutaneously into the skin of an immune-deficient mouse to check for the formation of teratomas consisting of tissues from all the three germ layers (Martin, 1981). However, the most stringent test of pluripotency is generation of chimeras and germline transmission. This is done by either aggregating ESCs with morula or by injecting ESCs into the blastocoel of the host blastocyst embryo. The pluripotent ESCs should then integrate with the cells from the host embryo and contribute to the formation of tissues from the three germ layers including germ cells to produce chimeras, which are capable of generating offsprings in order to show germline transmission (Bradley et al., 1984).

Table 2: Commonly used functional criteria to assess pluripotency

Assay	Experimental approach
In vitro differentiation	Differentiation induced cells are assayed for expression of various lineage markers
Teratoma formation	Formation of tumours demonstrating the potential to form cell types of all three lineages
Chimera formation	Contribution of cells to normal development
Germline transmission	Capability of the cells to develop functional gametes/germ cells

As mentioned previously, self-renewal is the division of a cell into two daughter cells where at least one is identical to the parent cell. ESCs possess both the properties of proliferating indefinitely and differentiating into all cell types (Smith, 2001). Maintenance of self-renewal and pluripotency depends on intrinsic determinants and extrinsic signalling (Chambers and Smith, 2004). Intrinsic factors are present in the pluripotent cells of an embryo where they regulate various developmental processes. However, extracellular signalling promoting self-renewal of ESCs in culture may be an *in vitro* phenomenon. This is probably because it is essential to provide nutrients for

proliferation of ESCs and inhibit ESCs from their natural tendency to differentiate in culture conditions. Thus, it is debatable whether the extracellular signalling performs the same function *in vivo*.

Both the various pluripotent cell types and the intrinsic and the extrinsic factors regulating self-renewal in ESCs have been discussed in the chapters 5, 6 and 7.

3. Using the rat in biomedical research

The laboratory rat, *Rattus Norwegicus*, was the first mammalian species to be used in the biomedical research. The first publication that used rat as an animal model dates back to 1856 where, French physician J. M. Philipeaux studied function of adrenal glands in albino rats (Philipeaux, 1856). Over the next 150 years, the rat became an ideal animal model for research in psychology, physiology, toxicology, cardiology, immunology and neuroscience. More than 800 different inbred, mutant and congenic rat strains have been created to mimic various human diseases. Due to its larger size in comparison to the mouse, a variety of procedures including *in vivo* imaging, surgery, cannulation and sampling of cerebrospinal fluid are easier to perform. Rat is the preferred model over mouse in retinal degeneration rescue studies as it is easier to carry out electroretinograms analysis in them (Vasireddy et al., 2011). Rats are also physiologically more similar to humans and provide an accurate representation of the phenotype for inflammatory disorders and neurodegenerative disorders in comparison to mice. In Huntington's disease studies, the death of striatal neurons is linked to the abnormal expansion of CAG repeat length in the *Huntingtin* gene. Rat models used to study this disease recapitulate the damage in the striatum with the similar CAG repeats as in the human mutation (von Horsten et al., 2003) where as mouse models require much longer repeats to mimic the human phenotype. In Parkinson's disease research, overexpression of alpha-synuclein in substantia nigra of rat models resulted in loss of dopaminergic neurons unlike the mice (Lo Bianco et al., 2004) (Yamada et al., 2004). Thus, there are several rat models that overcome the limitations of the mouse model to recapitulate the human diseases. They are more social and able to perform complex learning and memory tasks, which also make them an attractive tool in the study of learning, memory, cognitive, and motivational processes.

Despite the many advantages of rat over mouse models, mouse is still the predominant mammal in the experimental research. This is due to the longstanding existence of the core technologies for genetic modifications in mice for the past 30 years. Recently, different strategies have been developed to manipulate the rat genome and these will significantly enhance the rat genomic toolbox and enable the growth in genetic research using rats. The different gene targeting technologies have been outlined below.

4. Development of rat genetics

The predominance of the rat in scientific research is second only to humans and there are more scientific publications using rat models in comparison to any other model system according to Pubmed searches. Thus, establishment of different genetic tools to manipulate the rat genome are quintessential to further studies on various human diseases. In the preceding years, various different strategies using transgenics, siRNA knockdown and ethyl-nitrosyl urea (ENU) methodologies have been employed.

4.1. Mutagenesis via sperm manipulation

One prospective access point to manipulate the rat genome is the male gamete. One of the successful approaches applied in this context is the ENU mutagenesis. ENU is an alkylating agent that introduces single base pair substitutions to induce random point mutations (Cordes, 2005). It is estimated to create a functional mutation every 100 alleles, with 1 in every 1000, resulting in a phenotype (Augustin et al., 2005). Zan and colleagues generated the first knockout rat in 2003 by applying ENU chemical mutagenesis on gonads of male rats. ENU targeted the spermatogonial stem cells (SSCs). Mutagenized male rats of two inbred and an outbred strain produced F1 offsprings with mutations in breast cancer suppressor genes, *Brca1* and *Brca2* (Zan et al., 2003). Mutagenesis in rats using ENU can be utilized in a large-scale forward genetic screen where a large number of F1 animals can be generated by breeding the treated male rats with wild type female ones and screening them for desired phenotypes to identify gene mutations. It is a technically simple methodology to generate novel rat models and does not require any advanced genetic manipulations in oocytes or embryos and has the great advantage of automatic transmission of functional mutation through the germ line to the next generation. However, there are several limitations to this approach as well. First, it is very difficult to do genetic manipulation by gene targeting, as the mutations induced by ENU are random. Second, efficiency of mutagenesis is dependent on the dosage of ENU in different rat strains of various genetic backgrounds (van Boxtel et al., 2010). Third, a large number of F1 generation animals need to be maintained, to enable high-throughput screening, which can be costly and time-consuming. Lastly, the random point mutations induced by ENU occur throughout the whole genome, including unknown mutations in background producing different phenotypes. Hence, a methodology for targeting specific regions of genes would be a better approach to circumvent the above issues.

4.2. Transposon-mediated insertional mutagenesis

Transposons are mobile genetic elements, which can change their position within the genome. They require the presence of an active transposase enzyme to jump from one gene to another. Transposon-mediated gene-trap insertional mutagenesis in rodent models came into effect with the discovery and development of *Sleeping Beauty* (SB) transposon system in rats (Takeda et al., 2007) (Mátés et al., 2009). It was initially developed for random saturation mutagenesis in mice and then implemented for insertional mutations in rats (Kitada et al., 2009). The strategy behind the technique is to cause a null mutation by random insertion of a gene-trap transposon into a gene, disrupting the open reading frame of the gene, thus creating a transposon knockout mutation. Technically, it is performed by generating and breeding two transgenic rats, one carrying the gene-trap transposon and the other expressing the transposase vector, to create the so-called doubly transgenic "seed" male rats that carry both the elements. In the spermatogonia of these seed rats, the transposase catalyses the excision of one or more gene-trap transposons from the donor sites and inserts it into the new genomic site. The male "seed " rats are then mated with the wild type female rats resulting in the F1 generation containing only the insertion fragment which is caused by segregation of the insertion fragment from the transposase enzyme, thus immobilizing the inserted transposon (Jacob et al., 2010) (Huang et al., 2011).

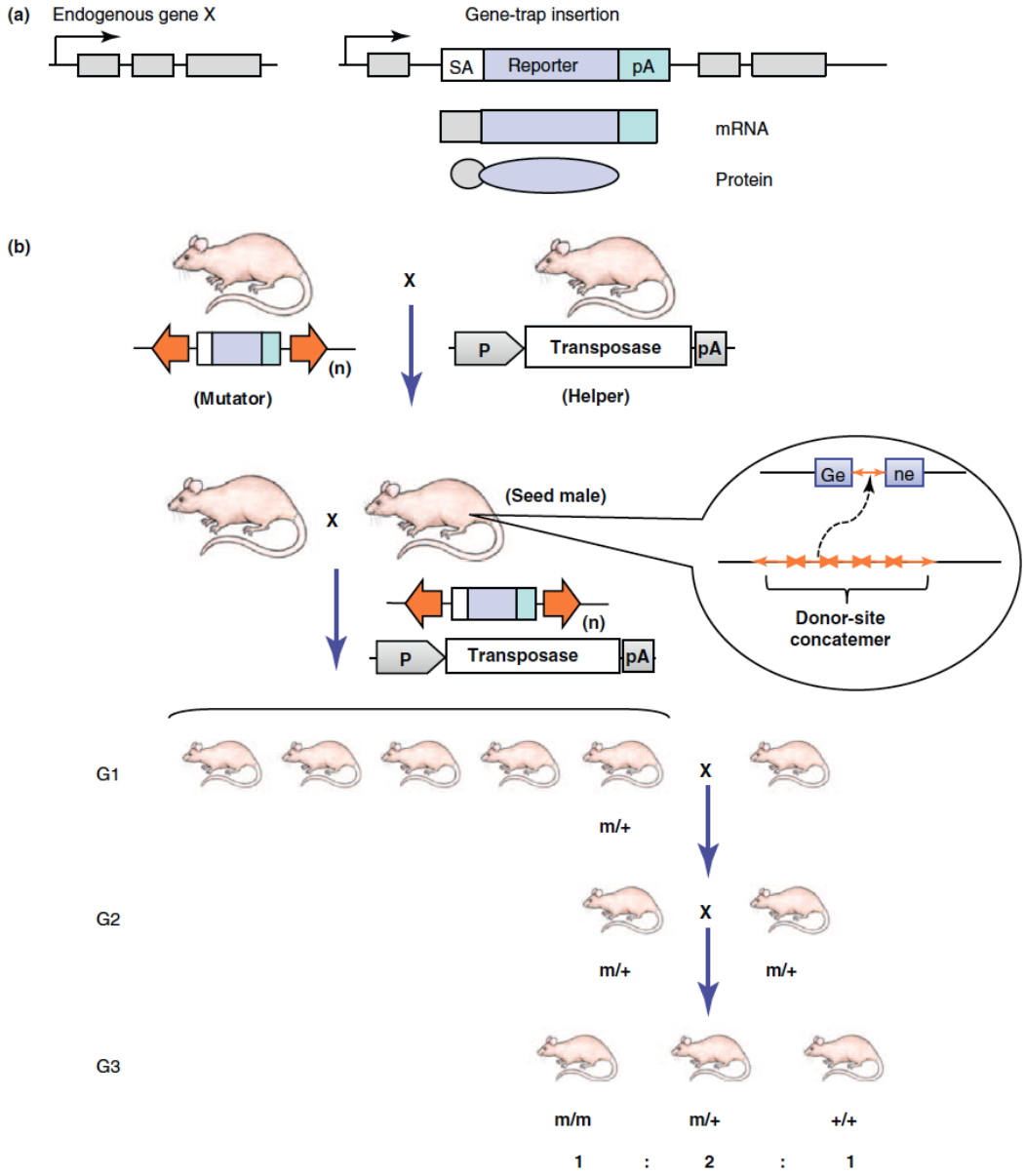

Figure 1: Diagrammatic representation of transposon-mediated mutagenesis

a) The gene trap cassette consisting of the reporter gene and polyadenylation tail is inserted into an endogenous gene disrupting the normal splicing of the gene. The mRNA is then translated into a fusion protein. b) The gene trap cassette is cloned into a transposon vector. Transgenic rat for gene-trap transposon is then bred with transposase harbouring transgenic rat to produce double transgenic rat "seed" male. In presense of transposase, transposons are mobilized and get inserted into genes in the developing spermatogonia. The seed males are later bred with wild type rats to generate G1 offsprings and screened for the mutations. Taken from (Jacob et al., 2010)

Various different techniques such as reporter gene expression or PCR of the gene-trapping cassette followed by breeding to obtain offsprings with single insertional sites can be used to identify the transposon mutants (Kitada et al., 2009). Thus transposon-mediated mutagenesis strategy can be used to generate various novel models. By introducing the transposase under the tissue specific promoter, one can also generate somatic mutations in the tissue of interest. This has been used to develop transgenic

mice to screen for cancer genes and modifiers within a specific tissue (Largaespada, 2009).

4.3. Zinc-finger nucleases

Zinc finger nucleases (ZFNs) were first implemented in 2009 to create knockout rats. The technique involved microinjecting mRNA or plasmid DNA encoding engineered ZFNs into the rat embryos (Geurts et al., 2009). ZFNs are artificially engineered proteins that combine multimeric zinc finger DNA-binding domains together with restriction endonuclease *FokI* cleavage domain. Each zinc finger motif is capable of recognizing and binding to triplets of DNA sequence in a sequence specific manner (Porteus and Carroll, 2005) and three to six zinc-finger motifs are combined to ensure target-sequence specificity. The *FokI* cleavage domain functions to cleave the target sequence. However, it must form a dimer to cleave double stranded DNA, thus requiring two ZFNs targeting a specific sequence (Geurts and Moreno, 2010). When two ZFNs are introduced into the cell, the dimerization of ZFNs at the recognition site generates a site –specific double strand break (DSB) in the chromosome. The cell, eventually repairs this DSB by either highly conserved homology-dependent repair (HDR) or non-homologous end joining (NHEJ) DNA repair pathway (Porteus and Carroll, 2005). Mostly, DSBs are repaired by NHEJ DNA repair pathway, which is less accurate than HDR. It occasionally results in the addition or loss of nucleotides at the DSB site resulting in a frameshift mutation, leading to a truncated and/or nonsense peptide, thus generating a gene knockout (Lieber, 2008). When DSBs are repaired by HDR pathway, a knock-in mutant can be generated. It is possible to provide the template in the form of an exogenous DNA fragment with homologous arms and the HDR mechanism results in the precise incorporation of the template (Cui et al., 2011). ZFNs can be used to produce a high number of heritable site-specific mutations in the rat by combining *in vitro* ZFNs DNA or mRNA with one-cell embryo via standard microinjection techniques. ZFNs mode of action in the earliest cell divisions leads to a high percentage of modified chromosomes in the resulting offspring, which can then be genotyped for the intended mutation (Geurts et al., 2010). Incase of off-target effects where ZFNs cause DSBs in untargeted loci, they can be easily mitigated by backcrossing, resulting in the loss of potentially undesired mutations. However, these off-target effects of ZFNs in genomic modification strategy in rats have not been found to date.

Recently, transcription activator-like effector nucleases (TALENs) have been used to generate a knockout rat by microinjection technique (Tesson et al., 2011). TALENs are engineered proteins created by fusion of transcription activator-like effectors with the catalytic domain of *FokI* nuclease. The mode of action is similar to ZFNs. However, the TALEN method still needs to be validated as a powerful tool for genetic modifications especially in rats, with only one paper published to date by Tesson *et al* (2011).

4.4. Embryonic stem cells

In mice, targeted knockout and knock-in by homologous recombination is most often performed in cultured ESCs, yielding thousands of genetically modified strains. In rats, the application of this ESCs-based technology is essential for generating knock-in models rather than the knockout ones, as there are many other strategies available for the latter purpose. The reasons for the delay to develop authentic ESCs in rats are related to the lack of maintained pluripotency in culture and germline-competent rat ESCs. Multiple groups had reported the derivation of rat ESCs lines. However, the identity of these so-called cell lines ranged from contaminating mESCs (Brenin et al., 1997) (Iannaccone et al., 1994) to cell lines with properties of extra-embryonic lineages (Vassilieva et al., 2000) (Buehr et al., 2003) (Iannaccone et al., 1994) (Stranzinger, 1996) (Ouhibi et al., 1995) and thus not capable of colonizing the germline of chimeras.

In 2008, Ueda *et al* (2008) reported the first putative rat ESCs that expressed the pluripotency markers; Nanog and Oct4 and maintained more than 40% normal karyotype over 18 passages. The rat ESCs were also capable of producing chimeras but failed to show germline transmission (Ueda et al., 2008). In late 2008, the use of two or three inhibitors (2i: MEK inhibitor PD0325901 and GSK3 inhibitor CHIR99021; 3i: FGF receptor inhibitor SU5402, MEK inhibitor PD184352 and GSK3 inhibitor CHIR99021) and a chemically defined basal culture media containing no fetal bovine serum was developed, which successfully established and maintained germline-competent rat ESCs (Buehr et al., 2008) (Li et al., 2008) (Ying et al., 2008). Addition of leukemia inhibitory factor (LIF) led to more stable culturing conditions for deriving rat ESCs. These rat ESCs expressed the pluripotency markers and were able to differentiate into all three germ layers *in vitro*. The establishment of 2i and 3i cell culture system has promoted the derivation of rat ESCs from various different rat strains including Sprague-Dawley (SD), Fischer 344 (F344), Dark Agouti (DA), Brown Norway (BN), Wistar (WI), Long-Evans (LE) and spontaneously hypertensive rats (SHR) (Buehr et al., 2008) (Li et al., 2008)

(Zhao et al., 2010) (Hong et al., 2012) (Tong et al., 2011). However, the ESCs derived from F344 strains have not been shown to be germline-competent (Hong et al., 2012). It has also been shown that the use of DIA-M cells or a mixture of MEFs and L-cells as feeder layers are optimal for deriving rat ESCs from SD and DA strains, respectively (Buehr et al., 2008) (Li et al., 2008). Another approach using an inhibitor cocktail media including 2i plus additional inhibitors of Rho-associated kinase (Y26732) and TGF-β signaling (A-83-01), collectively referred to as YPAC, was used to derive Wistar (WI), Long Evans Agouti (LEA) and hybrid Wistar/LEA ESCs. The additional inhibitors were used to prevent apoptosis and to enhance proliferation. The mouse ESCs medium containing 20% fetal bovine serum was used as basal cell culture media and MEFs were used as feeders in this study but LIF was not necessary. The majority of cell lines demonstrated chimerism and germline transmissions (Kawamata and Ochiya, 2010).

Rat transgenesis via genetic modification in ESCs, in 2010, marked the beginning of new era in rat genetics. Tong *et al.* (2010) created the first targeted gene knockout rat via homologous recombination. The DA ESCs derived in 2i+LIF conditions were used to target the tumour suppressor gene *p53*. Targeting efficiencies in the two ESC lines derived from DA were 1.12-3.70%. Many of the properly targeted cell lines developed chromosomal abnormalities and were polyploid. However, two of the 20 clones examined had euploid chromosome numbers and led to the successful generation of viable knockout (Tong et al., 2010). This has been a historic achievement validating the culminated efforts of many researchers to enable targeted genetic manipulation in rat ESCs. Using rat LIF instead of human LIF also paved the way for successful germline transmission of a transfected rat ESC line harbouring humanized Kusabira-Orange (huKO) gene by electroporation using the 2i+LIF culture medium (Hirabayashi et al., 2010). Previous studies have shown that rat ESCs cultivated in 2i+LIF medium were sensitive to electrophysical stimulation induced by electroporation causing cell death and the nucleofection method was found to be more efficient in gene introduction. It is possible that using rat LIF might be an option to keep these ESCs stable. Another study used the YPAC medium described earlier to generate *Oct4*-Venus transgenic rats in Wistar and LEA strains. The transgene in which the *Oct4* promoter transcribed the Venus gene was introduced by nucleofection into the rat ESCs. The transgenic animals were produced through germline transmission of the selected clones and no adverse effects were observed on chimera contribution due to gene introduction (Kawamata and Ochiya, 2010). Recently, protease activated receptor-2 (Par-2) knockout rat have also

been generated from DA rat ESCs in 2i medium (Yamamoto et al., 2012). However, the rate of germline transmission is really low in ESCs derived from DA, SD and Wistar rat strains (Tong et al., 2011). To date, only DA rat ESCs have shown to be germline competent after gene targeting by injecting the DA rat ESCs into F344 rat blastocysts but not into SD rat blastocysts (Tong et al., 2010) (Tong et al., 2011) (Hong et al., 2012). Thus, numerous challenges still remain in this field including the donor/host strain combinations for the efficient germline transmission from the chimeras.

4.5. Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are ESC-like cells, which have been derived from human, mice and rats by genetic reprogramming of the differentiated cells into a ground state of pluripotency (Lewitzky and Yamanaka, 2007).

Three different studies have yielded putative iPSCs from rats. Retroviral transfection of three mouse transcription factor genes (*Oct3/4, Sox2 and Klf4*) into hepatocyte progenitor cell line in presence of MEF feeder cells, along with the addition of three chemical inhibitors (PD0325901, A-83-01 and CHIR99021) and LIF to a defined culture media led to the successful isolation of iPSCs in this study. These progenitor cell-derived iPSCs yielded chimeric rats but none of the chimeric animals could demonstrate germline transmission (Li et al., 2009). Another approach used human reprogramming factor genes in combinations of three (*Oct3/4, Sox2 and Klf4*), four (*Oct3/4, Sox2, Klf4 and c-myc*) or five (*Oct3/4, Sox2, Klf4, c-myc and Nanog*) to transfect neural precursor and embryonic fibroblast cells in media containing FBS, PD0325901 and CHIR99021 and LIF on feeder cells derived from rat embryonic fibroblasts (Chang et al., 2010). In the third study, four transcription factors (*Oct3/4, Sox2, Klf4 and c-myc*) were transfected using a retrovirus to reprogram adult bone marrow and ear-tip fibroblasts under undefined culture conditions using MEFs but without LIF and chemical inhibitors (Liao et al., 2009). Both the studies suggested successful establishment of putative pluripotent rat iPSCs by gene expression studies and differentiation assays (Chang et al., 2010) (Liao et al., 2009). However, neither of the latter two studies demonstrated testing their iPSCs via blastocyst injections. No germline competency of rat iPSCs had been reported till 2011. In 2011, Hamanaka *et al.* (2011) for the first time reported generation of germline competent rat iPSCs. They transfected Wistar or DA rat embryonic fibroblasts with a lentiviral vector, carrying three mouse reprogramming factors (*Oct3/4, Sox2 and Klf4*) and seeded them on a layer of MEF feeders in a serum-free medium containing

inhibitors of MEK (PD0325901) and GSK3 (CHIR99021). The established rat iPSCs possessed all the key features of pluripotency. They showed expression of pluripotency markers Oct4 and Nanog, capacity to differentiate into all three germ layers, ability to produce chimeras with high efficiency and most importantly, contribute to germline transmission. High efficiency of germline transmission was obtained by injecting Wistar and DA rat iPSCs into Wistar blastocyst (Hamanaka et al., 2011). Thus, the successful generation of germline- competent rat iPSCs has opened the doors to the tremendous utility of these cells for directed differentiation and phenotyping *in vitro,* as well as preclinical modeling strategies for tissue generation and organ repair.

5. Pluripotent cell types of a mouse

Embryonic Stem cells (ESCs) are derived from the preimplantation embryo of a mouse. Investigations into the nature of pluripotency have lead researchers to attempt the derivation of pluripotent cells from alternative sources. I have outlined below the various types of pluripotent cell types derived from mice till date.

5.1. Embryo-derived stem cells

Embryo derived stem cells can be obtained from pre- and early post-implantation stages of an embryo. They are categorized into embryonic stem cells, embryonic germ cells and epiblast stem cells.

5.1.1. Embryonic Stem Cells (ESCs)

5.1.1.1. Origin and derivation of embryonic stem cells

The striking observation that the early mouse embryos engrafted into adult mice produced teratocarcinomas, containing a significant population of undifferentiated cells paved the way for the establishment of embryonic stem cells (Stevens, 1970) (Solter et al., 1970). Co-culturing the embryonal carcinoma (EC) cells with mitotically inactivated embryonic fibroblasts and fetal calf serum not only promoted efficient establishment of EC cells but also increased their differentiation capacity (Martin and Evans, 1975) (Martin et al., 1977). Thus, these fibroblasts, which were providing some important factors, were described as feeder cells. The undifferentiated EC cells contributed to multilineage differentiation when injected into the host blastocyst to form chimeras. Martin and Kaufmann then went on to investigate culturing primary embryo cells in the presence of feeders. They termed these cells as embryonic stem cells to distinguish them from the EC cells. Thus the first ESCs were successfully derived from blastocyst in 1981 in the presence of serum and feeders. Embryos at the late blastocyst stage were plated intact on the feeders with serum for a few days before dissociating them into single cells (Evans and Kaufman, 1981) (Martin, 1981). ESCs closely resemble EC cells in accordance with the formation of teratomas which contain tissues from all three germ layers i.e. ectoderm, mesoderm and endoderm (Kaufman et al., 1983). Various techniques can be used to introduce ESCs into the preimplantation embryo where they contribute to all fetal lineages plus the yolk sac mesoderm, allantois and amnion but poorly to extraembryonic endoderm and almost never to trophoblast (Bradley et al., 1984) (Beddington and Robertson, 1989). Unlike EC cells, ESCs have the ability to integrate

into the embryo and produce viable chimeras. In contrast to EC cells, they have a diploid karyotype, which is necessary for meiosis and gives them the ability to generate germ cell lineage and thus promote germline transmission (Bradley et al., 1984). Another surprising feature of ESCs is that the majority of the ESC lines are 40, XY. The XY genotype confers a huge advantage over XX for establishment of germline transmission as XX ESCs have both their X chromosomes active suggestive of a drawback for ESC propagation (Rastan and Robertson, 1985). Male chimeras produce more offspring in comparison to female chimeras and also convert undefined genital ridges into male gonad development. As a result, all spermatocytes are of ESC origin (Bradley et al., 1984).

For many years, ESC derivation was poorly understood and it became necessary to refine and define the conditions required for efficient generation of ESCs. In 1988, two different studies by Smith and Williams showed that the essential self- renewal function of feeder cells arose from cytokine produced by these cells termed as Leukemia inhibitory factor (LIF) (Williams et al., 1988) (Smith et al., 1988). Lately, serum has been replaced by the addition of bone morphogenetic protein 4 (Bmp4) consequently allowing the propagation of germline competent ESCs by supplementation with the two cytokines LIF and Bmp4 (Ying et al., 2003). However, these conditions still did not lead to derivation of true ESCs from non-permissive strains like CBA. Interestingly, the efficiency of ESCs derivation was dependent upon the genetic background of the embryo; 129 strain was found to be most permissive where as ESCs derivation from CBA strain was not possible (Batlle-Morera et al., 2008) (Buehr and Smith, 2003). This was reasoned with the strain specific variation of Erk signalling. Erk pathway is independently activated by FGF4 and LIF (Batlle-Morera et al., 2008) (Wray et al., 2010). In 1999, Burdon *et al.* showed that the inhibition of Erk pathway promoted self-renewal of ESCs by blocking differentiation cues provided by it (Burdon et al., 1999b). Inhibition of Erk also improved the efficiency of ESC derivation from non-129 strains of mice (Batlle-Morera et al., 2008) (Buehr and Smith, 2003). The embryonic diapause property of rodents also facilitates the derivation of ESCs. Diapause is a phenomenon, which occurs in mice and rats when the suckling mother produces embryos. In this situation, the embryos develop to the blastocyst stage, hatch from zona pellucida and segregate into the epiblast and the hypoblast but are not implanted until oestrogen is restored. The epiblast retains the expression of Oct4 and Nanog and has both X chromosomes active in female embryos (Silva et al., 2009). These embryos can regain development

even after 3-4 weeks of implantation delay. LIF signalling has been shown to be very crucial for prolongation of the epiblast lifespan during this period thus creating a close relationship between early epiblast and ESCs (Nichols et al., 2001). ESCs were first derived from the blastocyst in diapause. Thus, it has been proven that LIF enhances the efficiency of ESC generation (Brook and Gardner, 1997).

Recently, small molecules, which inhibit specific kinases have become a great tool in establishing a new protocol for derivation and propagation of ESCs. This new cell culture regime termed 2i specifically inhibits 2 kinases: mitogen activated protein kinase/ Erk kinase and glycogen synthase kinase 3 (Fig. 2) (Ying et al., 2008). Most importantly, inhibition of Erk signalling in the pre-implantation stage embryo abrogates the differentiation processes required for the development, henceforth preventing the formation of hypoblast (Nichols et al., 2009). However, ESCs derivation is most efficient only when GSK3β is inhibited or LIF/Stat3 pathway is activated. Using small molecules for ESC derivation has made it possible to efficiently generate germline competent ESCs from both the permissive and non-permissive strains of mice as well as few strains of rat.

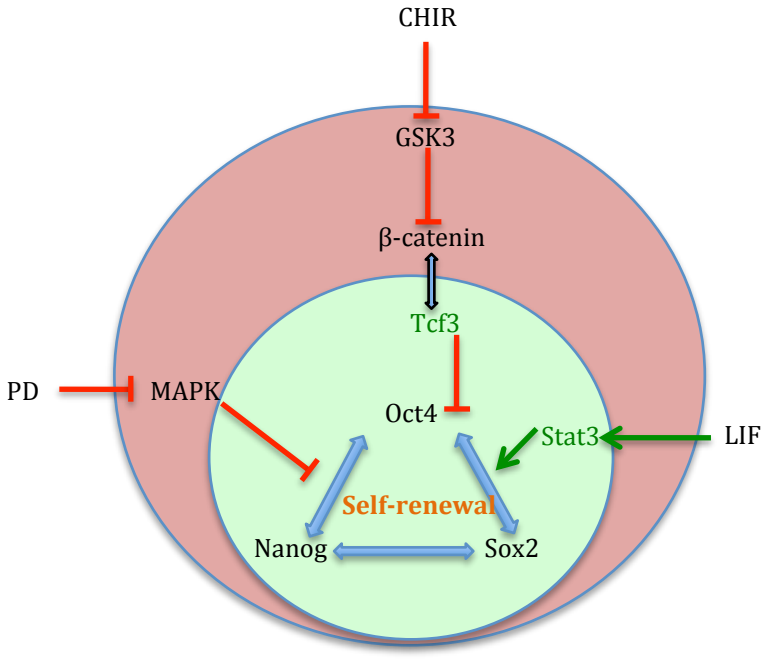

Figure 2: Development of "2i" for ESCs propagation

Combination of GSK3 inhibitor (CHIR), MEK inhibitor (PD) and LIF supports successful derivation and propagation of ESCs. Inhibition of GSK3 stabilises the intracellular β-catenin, which interacts with Tcf3 and ablates its repressive function on pluripotency factors (Wray et al., 2011). Inhibition of MEK blocks the differentiation of ESCs and LIF supports self-renewal by activating Stat3 and both positively affect the pluripotency network.

5.1.1.2. Relationship between pre-implantation stage embryo and embryonic stem cells

In the mouse, ESCs are derived from the early epiblast of E3.5 blastocyst. It is essential to understand the development of an embryo to the blastocyst stage and genes regulating this process in order to comprehend the relationship between pluripotency and ESCs derived.

Three different cell compartments are present in the blastocyst stage embryo: the trophoectoderm which produces the trophoblast/placenta and the chorion, the primitive endoderm/hypoblast which contributes to the yolk sac and the epiblast which forms the embryo proper, the umbilical cord and the amnion (Ralston and Rossant, 2005). Two distinct lineage segregations: the trophoectoderm and the inner cell mass (ICM) segregation and the epiblast and the hypoblast segregation in the ICM leads up to the formation of blastocyst (Fig. 3).

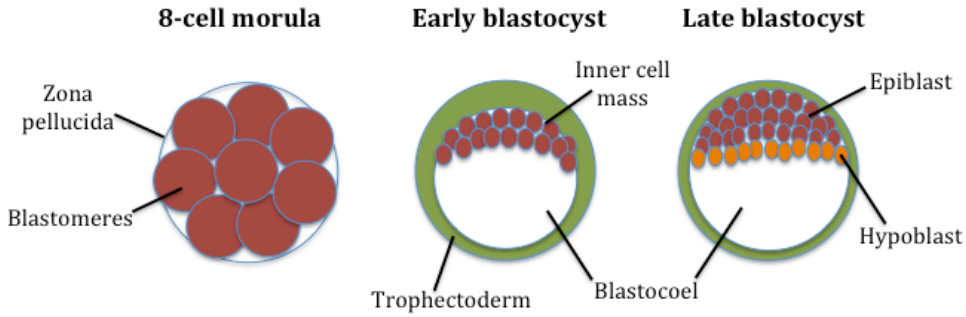

Figure 3: Different segregations of a pre-implantation embryo

The first lineage segregation occurs from late morula to early blastocyst where the outer cells form the trophectoderm and the inner cells form the inner cell mass and form a cavity called blastocoel. The next differentiation event occurs at the late blastocyst stage when inner cell mass segregates into epiblast and the hypoblast

Fertilisation of the oocyte produces a zygote, which after three rounds of cleavage division leads to the formation of morula. The POU domain transcription factor Oct4 (also known as Oct3/4 or POU5F1) is expressed throughout the embryo till the blastocyst stage.

The first segregation- trophectoderm and ICM segregation occurs at the late morula stage when cell compaction, a process where the blastomeres become closely apposed to one another, takes place. Another gene, *Cdx2*, encoding caudal related transcription factor comes into play at this stage. The outer layer of the cells forms an epithelial structure called trophoectoderm, which creates a boundary around the other compacted cells and secretes proteins promoting the formation of an inner cavity or blastocoel, which pushes the ICM to one side. The embryo is now called the blastocyst and consists

of the ICM and the trophectoderm. *Oct4* and *Cdx2* act as the selector genes for ICM and trophectoderm and act antagonistically to each other to regulate the cell fates (Strumpf et al., 2005). Recently, TEA DNA binding domain/transcription enhancer factor (TEAD/TEF) family transcription factor TEAD4 has been shown to regulate the expression of Cdx2 in pre-implantation stage embryo. It acts in conjunction with its co-activator protein YAP to induce the expression of the trophectoderm genes (Nishioka et al., 2009).

Next differentiation event occurs at the late blastocyst stage, in mouse at E4.5, just before hatching from the zona pellucida where the ICM segregates into the epiblast and the hypoblast/primitive endoderm. The primitive endoderm forms an epithelial cover facing the blastocoel around the epiblast (Fig. 3). Prior to the segregation, Oct4 positive ICM has heterogenous constitution and consists of cells expressing Nanog and other cells expressing Gata6. These are the cells, which later on become the precursors of epiblast and hypoblast respectively. Thus Nanog and Gata6 act as the selectors for the above lineages (Chazaud et al., 2006). In agreement with their *in vivo* expression pattern, Oct4 and Nanog are also expressed in the pluripotent ESCs where as Cdx2 and Gata6 are not. This suggests that ESCs correspond to the epiblast cells beyond the development of trophectoderm and primitive endoderm.

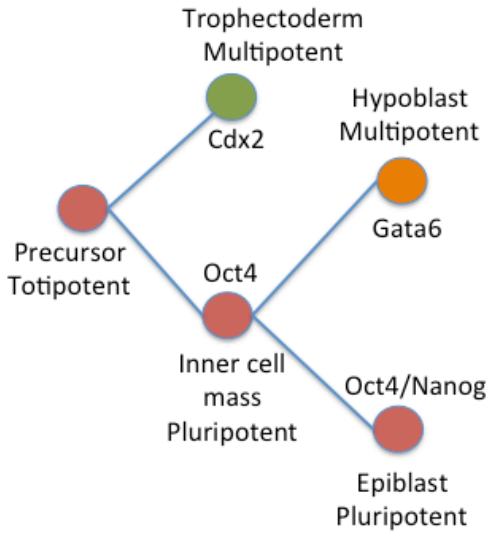

Figure 4: Different gene expression patterns *in vivo*

a) Different gene expression patterns during blastocyst formation. Oct4 and Nanog both are expressed in all cells till late morula stage and then become restricted to the inner cell mass and epiblast of the blastocyst. Cdx2 is expressed in the outer cells of the blastocyst forming the trophectoderm. Gata6 is expressed in the primitive endoderm or hypoblast of the late blastocyst stage.

b) Selector genes for lineage decision. Oct4 and Cdx2 act antagonistically to segregate into inner cell mass and trophectoderm lineage. Similarly, Nanog and Gata6 expression leads to the second segregation within the inner cell mass. Modified from (Ralston and Rossant, 2005)

5.1.2. Epiblast stem cells

Epiblast stem cells (EpiSCs) are pluripotent stem cell lines established from both mouse and rat post-implantation embryos. They are derived from E5.5-E6.5 embryos and cultured in the medium supporting derivation and expansion of human ESCs, hence cultured in medium containing activin and bFGF (Tesar et al., 2007) (Brons et al., 2007). Upon blastocyst injection or morula aggregation, the capacity of EpiSCs to integrate into the pre-implantation stage mouse embryo was really low leading to extremely low contribution towards chimera production. It can possibly be explained by the restricted capacity of these cells to develop into early cell lineages. However, they express the pluripotency markers Oct4, Sox2 and Nanog and are capable of multi-lineage differentiation proven by formation of teratomas containing a wide variety of tissues like muscle, cartilage, liver, gut and neuronal rosettes (Tesar et al., 2007) (Brons et al., 2007). Unlike mESCs, EpiSCs do not have reactivation of the X chromosome and express genes like FGF5 and Nodal. They have no expression of genes like Rex1 and Gbx2, which are specific markers of inner cell mass and ES cells. Although, they are pluripotent, EpiSCs have shown to differentiate in conditions required for self-renewal and pluripotency of ES cells. They spontaneously differentiate into the somatic cell lineages like endoderm. Thus, due to their inclination towards differentiation, the self-renewing state of EpiSCs is said to be primed pluripotent state rather than naïve one as of the mESCs. In 2009, Guo *et al.* reported the conversion of naïve to primed pluripotent states by culturing ESCs in medium containing activin and bFGF to form EpiSC-like cells which had an inactive X chromosome and same gene expression pattern like embryo-derived EpiSCs (Guo et al., 2009). It has recently been possible to convert EpiSCs to ES-like cells by introducing one of the transcription factors Klf4, Klf2, Nanog, cMyc, Nr5a1 or Nr5a2 to 2I and LIF or serum and LIF on feeders (Guo et al., 2009) (Guo and Smith, 2010) (Hall et al., 2009) (Silva et al., 2009) (Hanna et al., 2009). These EpiSC derived ES-like cells behave as ESCs and contribute to chimera formation and germline transmission.

It is of great interest to study EpiSCs because they are very similar to human ESCs. They both are derived and cultured in activin and bFGF, have an inactive X chromosome, flattened morphology, cellular heterogeneity and capacity to form teratomas (Tesar et al., 2007) (Brons et al., 2007) (Shen et al., 2008) (Vallier et al., 2005). Human ESCs cannot undergo the stringent test of pluripotency for formation of chimeras due to ethical issues. The fact that the preimplantation stage of human development is closer to the epiblast stage of mouse development suggests that human ESCs are in a less naïve

state and henceforth more similar to the primed state of EpiSCs (Nichols and Smith, 2009). It also provides us with the intriguing possibility of capturing human ESCs in their naïve state and studying the molecular mechanisms behind the pluripotency of human ESCs.

5.1.3. Embryonic Germ Cells

Embryonic Germ Cells (EGCs) are pluripotent stem cells derived from primordial germ cells (PGCs) of the post-implantation embryo E7.5- E13.5. EGCs are derived from PGCs by culturing *in vitro* in the presence of stem cell factor (SCF), LIF and basic fibroblast growth factor (bFGF) (Matsui et al., 1992). Recently, EGCs can also be derived either by culturing PGCs in traditional medium followed by cultivating them later in 2i+LIF medium or directly generating them in 2i+LIF medium on SCF null feeder cells (Leitch et al., 2010).

PGCs are progenitors of the germ cell lineage, which contribute to the development of healthy gametes in the adult. PGCs arise as alkaline phosphatase positive cells at the base of allantois at 7.5dpc. At E8.5, they migrate and are found in the hindgut endoderm from where they later associate with dorsal mesenteries and translocate to genital ridges by 10.5dpc (CHIQUOINE, 1954) (Ginsburg et al., 1990). Transcriptional similarities between ESCs and PGCs are maintained between E8.5 to E13.5 when they express some key pluripotency genes like Sox2, Nanog and Oct4 (Kurimoto et al., 2008) (Yamaguchi et al., 2005). Besides transcriptional changes, global epigenetic reprogramming events also occur. The inactive X chromosome starts to activate and is completely active by E14.5. Furthermore, histone modifications occur in the migrating PGCs where there is a decrease in histone 3 lysine 9 dimethylation (H3K9me2) for gene induction and increase in H3K27me3 for gene repression between E7.5 and E8.5 (Hajkova et al., 2008) (Seki et al., 2005). From E10.5, PGCs start to colonize the gonads and involve genome wide DNA demethylation of imprinted genes and chromatin remodeling in order to reset the epigenome of germ line. Thus, mouse EGCs derived from E11.5-E12.5 PGCs lacking methylation imprints contribute to chimeras with abnormalities in contrast to the ones derived from E8.5 to E11.5.

5.2. Induced pluripotent stem cells (iPSCs) and Reprogramming

Reprogramming is the process of altering the epigenetic marks of the cell to change its developmental potency. Induced pluripotent stem cells are embryonic stem like cells that are derived by epigenetic reprogramming of a somatic cell to a pluripotent state. There have been several approaches towards efficient and successful reprogramming for decades. Cloning by somatic cell nuclear transfer technique was the very first technique in the history of reprogramming shown by John Gurdon in Xenopus(GURDON, 1962) and few decades later by Ian Wilmut in sheep (Wilmut et al., 1997), which demonstrated that the epigenetic state of fully differentiated somatic cells is reversible and can be reprogrammed to an embryonic state. Further on, the fusion of somatic cells with embryonic stem cells (ESCs) or embryonic germ cells (EGCs) or exposure to ESCs or embryonic carcinoma extract was also shown to be a method for reprogramming (Taranger et al., 2005). But, Takahashi and Yamanaka achieved the major breakthrough in 2006, when they showed that somatic cells could be reprogrammed by inducing the forced expression of four transcription factors – Oct4, Sox2, Klf4 and cMyc via retroviral transduction,. These cells expressed the ESC markers and could contribute to chimera generation and were thus designated as induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). However, cMyc, one of the transcription factors used in reprogramming, is an oncogene, which frequently produces tumors in iPSCs chimeric mice and it would be better to obviate it from the reprogramming process. Wernig *et al*, in 2008, showed that cMyc is dispensable for reprogramming (Wernig et al., 2008). Recently, Yamanaka's group discovered Glis1, which belongs to the GLI family transcription factor, present in unfertilized eggs and oocytes and can replace cMyc in the reprogramming process thus decreasing the tumorigenicity (Maekawa et al., 2011). Nonetheless, in order to completely circumvent the issue of tumor formation and to bring iPSCs use in clinical settings, it is essential to look into viral and transgene free reprogramming approaches. Different non-integration and removable systems include episomal vectors, piggybac transposon system and minicircle vectors. Although, these systems are detachable, they still involve the introduction of foreign DNA into the cell, which may cause genomic alterations. Thus, inducing the expression of the reprogramming factors in their protein form is highly desirable. This has been shown by fusing reprogramming factors with cell-penetrating peptides and exposing fibroblasts to these recombinant proteins (Zhou et al., 2009) and more recently, by using modified mRNA encoding reprogramming factors (Warren et al., 2010). However, these

techniques are expensive and difficult to replicate. Recent studies have also shown that microRNAs (miRNA) alone are able to reprogram somatic cells to pluripotent state. miRNA application has a great advantage over other methods as it avoids using oncogenic factors and does not involve introducing genetic changes into the genome (Lin et al., 2008) (Anokye-Danso et al., 2011) (Miyoshi et al., 2011). However, the molecular mechanism on how miRNA activates pluripotency pathways is still unknown.

Thus, there are still a significant number of hurdles to overcome like efficient reprogramming protocols, transplantation safety and immunogenecity before bringing iPSCs to the clinics.

6. Intrinsic determinants of ESC self-renewal

Several transcription factors have been shown to play essential roles in both early development and maintenance of pluripotency in ESCs. They are regulated by themselves and the extrinsic signals described later. In the presence of these genes, the self-renewal genes are activated and the differentiation genes are repressed. The core transcription factors: Oct4, Sox2 and Nanog are described below.

6.1. Oct4

Oct4 is one of the key components of the molecular circuitry regulating embryonic stem cell proliferation and differentiation. It belongs to Octamer class of transcription factors that binds to an octamer sequence, ATGACCT (Falkner and Zachau, 1984) (Parslow et al., 1984). Along with Pit and Unc, Oct proteins define the POU class of proteins that have two DNA binding domains: POU-specific domain and POU homeodomain. During mouse embryo development, Oct4 is expressed in the unfertilized egg, in all the cells of an embryo prior to segregation of ICM, and the ICM. It is also expressed in the epiblast of pre- and post-implantation stage embryo and later becomes restricted to migrating PGCs.

Oct4 deficient mouse embryos develop to a stage that look like blastocysts but are developmentally compromised. These embryos when allowed to attach *in vitro* and grow, form colonies that are only comprised of trophoectoderm cells (Nichols et al., 1998). As these structures do not have a genuine ICM, they cannot produce ESCs. Therefore, Oct4 has been shown to be crucial for preventing trophectoderm differentiation and maintaining pluripotency during embryonic development. In mouse ESCs, loss of Oct4 causes differentiation into trophectoderm. Overexpression of Oct4 by 150% results in differentiation into primitive endoderm and mesoderm indicating that a relative amount of Oct4 determines the cell fate and precise levels of this gene are required in order to maintain ESC state (Niwa et al., 2000). Little is known about the upstream regulators of Oct4. Oct4 contains conserved distal and proximal enhancers on its promoter that can either activate or repress its expression depending on the binding factors (Pan et al., 2002). Interestingly, Nanog can activate Oct4 expression as well (Pan et al., 2006).

Several target genes of Oct4 have been identified. Few of them are FGF4, Rex1, Utf1, Fbx15, Opn and Sox2. LIF/STAT3 pathway (discussed later) is an important pathway promoting ESC self-renewal. However, LIF does not seem to regulate Oct4 and Oct4 does

not appear to regulate JAK/STAT signalling. Thus, Oct4 functions parallel to the LIF pathway.

6.2. Sox2

Sox2 is a member of a high mobility group (HMG) box DNA binding domain proteins. It is defined by the relationship of HMG box to the testis-determining factor SRY and thus the name Sox (Sox, SRY HMG box) (Bowles et al., 2000). It plays a role in regulating transcription and chromatin architecture.

Along with Oct4, it forms a complex on the enhancer DNA sequence of *FGF4* and acts synergistically to stimulate transcription (Ambrosetti et al., 2000). This interaction allows Sox2 to play a role in the regulation of ICM and its derivatives. It is also present in ESCs and neural stem cells. Sox2 null embryos do not have an epiblast, but this condition can be rescued by injecting wild type ESCs into the Sox-/- blastocysts. Unlike Oct4, outgrowths of Sox2-/- embryos generate colonies consisting of both trophectodermal and primitive endodermal cell types (Avilion et al., 2003).

6.3. Nanog

Nanog is a homeodomain containing transcription factor, which maintains ESCs pluripotency independent of LIF. Wang *et al.* (2003) first described this gene as an ENK (early embryo NK) gene expressed in ESCs (Wang et al., 2003). However, its function was unknown then. It was later recloned and renamed as Nanog by Mitsui *et al.* and Chambers *et al.* independently in 2003. Using *in silico* differential expression analysis and functional cDNA expression cloning, Mitsui *et al.* and Chambers *et al.* respectively, identified Nanog as the transcription factor maintaining ESCs pluripotency independent of LIF-STAT3 pathway (Mitsui et al., 2003) (Chambers et al., 2003). Nanog expression is first detected in the inner cells of the compacted morulae then in the ICM of the blastocyst stage embryo. It further gets restricted in the epiblast. Post-implantation, it can be detected in primordial germ cells of the genital ridges. Nanog mRNA is enriched in ESCs, EGCs and ECs and not present in adult tissues (Chambers et al., 2003).

Upon differentiation, Nanog expression is downregulated. Physiological levels of Nanog do not prevent differentiation of ESCs upon LIF withdrawal thus making it one of the transcription factors expressed in the pluripotent cells, which disappears upon differentiation. Nanog-/- embryos develop a blastocyst without an epiblast. It is possible to derive Nanog-/- ESCs from the embryos but they differentiate slowly into extra-embryonic endoderm lineages as evident from the absence of the epiblast (Mitsui et al.,

2003). Therefore, Nanog is essential for the maintenance of epiblast. Overexpression of Nanog in ESCs could maintain the cells pluripotent even in the absence of LIF. In cells overexpressing Nanog, the phosphorylation levels of STAT3 remain unchanged and similarly, Nanog levels are not affected with altered Stat3 signalling. Inhibition of Jak, an upstream activator of Stat3 also does not modulate the levels of Nanog. Thus, it can be said that Stat3 does not induce the expression of Nanog and Nanog does not regulate Stat3 (Chambers et al., 2003). Interestingly, it has also been shown that Nanog physically interacts with SMAD1, thus inhibiting the activity of BMP signalling. Henceforth, balancing ESC pluripotency and differentiation by negative feedback mechanism between the two (Suzuki et al., 2006). This suggests that Nanog maybe a downstream effector for extrinsic factors (Chambers et al., 2003).

6.4. Combinatorial signalling between the core pluripotency factors: Oct4, Sox2 and Nanog

One of the most studied and key interacting partners of Oct4 is the Sox2. Sox2/Oct4 complex has been shown to be important in regulating many genes required for embryogenesis and for ESC self-renewal and pluripotency. The best characterized target of Oct4 remains to be FGF4. In 1995, Yuan *et al.* identified closely spaced binding sites for Oct4 and Sox2 on the FGF4 enhancer and showed that the two transcription factors acted synergistically to activate the transcription of *FGF4* (Yuan et al., 1995). Since then, it has been shown that all of the Oct4 downstream targets have oct/sox heptamer element in the promoter which promotes the binding of Oct4 and Sox2 and triggers the transcription of the genes like Utf1 and Nanog (discussed below in detail) (Nishimoto et al., 1999) (Rodda et al., 2005). Oct4 and Sox2 also reciprocally regulate their own expression. Both *Pou5f1* and *Sox2* contain oct/sox elements that bind to Oct4 and Sox2. Disruption of their own oct/sox cassettes lead to a strong reduction in the activity of Oct4 and Sox2 promoters in ESCs and ECs (Chew et al., 2005) (Tomioka et al., 2002) (Okumura-Nakanishi et al., 2005). Thus, Oct4 and Sox2 work together in maintaining the expression of essential transcription factors through autoregulatory and multiple component loop network motifs.

Deletion of Sox2 in ESCs triggers their differentiation similar to Oct4 deletion. However, enforced expression of Oct4 in these cells can rescue both their ability to self-renew and to differentiate into derivatives of all three germ layers. Thus, it can be concluded that the key contribution of Sox2 is to maintain the level of Oct4. Masui *et al.* showed that

Sox2 regulates Oct4 transcription by upregulating Nr5a2, which activates Oct4 and by downregulating Nr2f2, which represses Oct4 transcription. They also showed that expression of Oct/Sox target genes was not affected by the loss of Sox2 probably because of the compensatory effect by expression of other Sox family members, Sox4, Sox11 and Sox15 in these ESCs (Masui et al., 2007). In addition to these knockout studies, Sox2 overexpression studies showed a negative regulatory loop concerning the Oct4/Sox2 target genes. It was shown that overexpression of Sox2 not only inhibited the activity of its own promoter but also the activity of Oct4, FGF4, Nanog and Utf1. Surprisingly, the inhibitory effect observed with Sox2 was not observed with Oct4. This led to the hypothesis that overexpression of Sox2 in ESCs triggers their differentiation (Bernadt et al., 2004) (Boer et al., 2007). It is obvious from the findings above that Oct4 and Sox2 behave as molecular rheostat in maintenance of ESC self-renewal and pluripotency.

Recently, researchers found out that Nanog is a target of Oct4/Sox2 complex. The promoter of Nanog has an oct/sox element, which promotes the binding of Oct4 and Sox2 to it and promotes the transcription and regulation of Nanog (Kuroda et al., 2005) (Rodda et al., 2005). It has been shown that high levels of Nanog are beneficial to ESC self-renewal. However, overexpression of Nanog in cells that lacked both endogenous Oct4 alleles and were cultured by doxycycline regulated Oct4 transgene, could not reverse the differentiation that occurred due to repression of Oct4 by presence of doxycycline. This confers the requirement of Oct4 in Nanog mediated self-renewal. However, Nanog expression could be readily detected even in Oct4 deficient embryos, which implies that Oct4 is not essential for expression of Nanog and there are other pluripotency factors contributing to the regulatory mechanisms for Nanog (Chambers et al., 2003).

In a recently published paper by Pan *et al.*, a negative feedback loop formed by Nanog, Oct4 and another pluripotent factor FoxD3 is described. Oct4 maintains Nanog expression by directly binding to its promoter when present below steady state but represses the expression of Nanog when above the normal level. Instead, FoxD3 positively regulates Nanog to counter the inhibition by excess Oct4. Conversely, Nanog and FoxD3 function as activators of Oct4 and when the Oct4 level goes above the steady state, Oct4 represses its own promoter and Nanog exerts a negative feedback loop on its regulation thus maintaining ESC state (Fig. 5) (Pan et al., 2006).

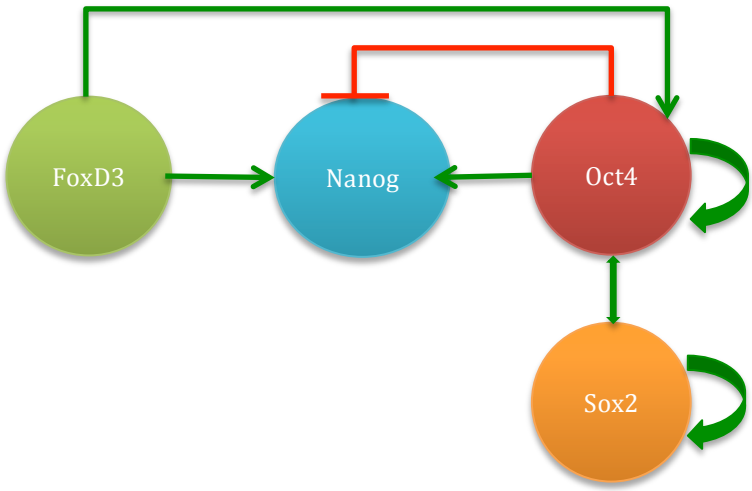

Figure 5: Illustration of the interconnection between core pluripotency factors

The core regulatory network of ESCs is centered on Oct4 and Sox2 which are linked to Nanog, which is individually dispensable but collectively sustain pluripotency of ESCs. Oct4 and Sox2 autoregulate their expression and contribute to the transcription of Nanog. FoxD3 positively regulates the expression of Nanog to repress the inhibition by excess Oct4.

Overall, this study suggests that the key pluripotency factors always work together to regulate both their expression and those of their target genes in order to maintain the key properties of ESC self-renewal and pluripotency.

7. Extrinsic determinants of self-renewal

Three distinguishing characteristics of embryonic stem cells are pluripotency, self-renewal and indefinite cell proliferations. Several exogenous factors are involved in modulating these traits both *in vivo* and *in vitro*. The most important pathways regulating self-renewal in mESCs are those mediated by the cytokine LIF. Recently, Wnt canonical pathway has also been shown to play a role in maintenance of pluripotency. Reciprocally, it also plays a critical role in initiation of the lineage commitment. These different outcomes have created enormous controversies concerning Wnt signalling. The role of LIF signalling cascade and Wnt pathway in regard to maintenance of self-renewal and pluripotency in mESCs is summarised below.

7.1. LIF signalling pathways

LIF belongs to the interleukin-6 (IL-6) cytokine family, which was initially identified by its activity to inhibit proliferation and induce differentiation of mouse myeloid leukemia cells (Tomida et al., 1984) (Gearing et al., 1987). It was later discovered that the mESCs can be established and maintained in the absence of feeder cells in conditioned medium prepared from Buffalo rat liver cells. The component of conditioned medium responsible for inhibition of differentiation in mESCs was named as DIA (Smith et al., 1988) (Williams et al., 1988). Eventually, investigators realized that the two proteins LIF and DIA were identical.

LIF activates signal transduction from cell surface receptors. The LIF receptor consists of LIF-specific receptor subunit LIFRβ and the common signal tranducing protein gp130 (Davis et al., 1993). LIF binds directly to the LIFRβ that subsequently heterodimerizes with gp130 forming a trimeric complex triggering three different signalling pathways: the JAK (Janus Kinase)/STAT3 (signal transducer and activator of transcription 3) pathway; the PI3K (phosphoinositide 3 kinase)/AKT pathway; and SHP2 (SH2 domain containing tyrosine phosphatase 2)/MAPK (mitogen activated protein kinase) pathway (Fig. 6).

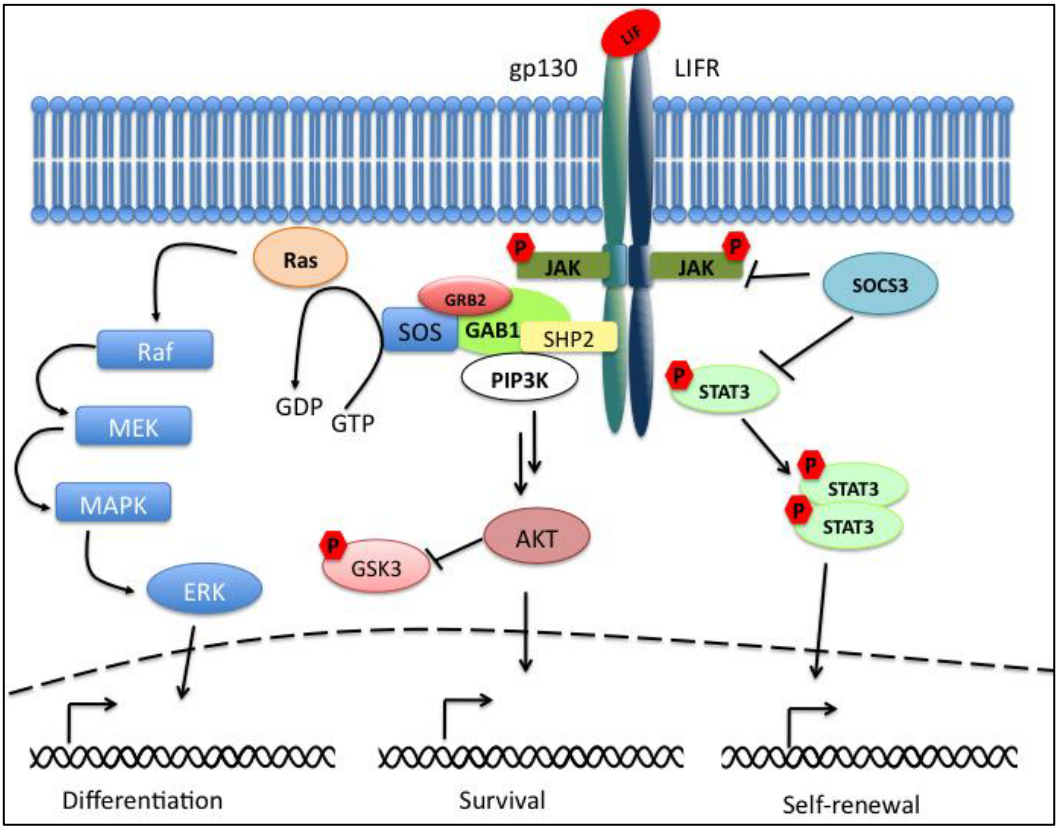

Figure 6: LIF signalling cascade

LIF/JAK/STAT3 and LIF/PI3K/AKT pathway are involved in maintenance of self-renewal and pluripotency. LIF/SHP2/MAPK pathway is involved in differentiation processes. (Graf et al., 2011)

7.1.1. LIF/JAK/STAT3 signalling

The tyrosine kinase JAK is constitutively bound to the gp130 in its inactive form. Upon LIF binding, LIFRβ recruits gp130 to form a heterodimer, which activates JAK through transphosphorylation within a single JAK or between two JAKs (Burdon et al., 2002). Among the four JAKs known, JAK1 and JAK2 are the important ones involved in the LIF signalling pathways. Activated JAKs phosphorylate the tyrosine residues on intracellular domain of gp130, which then act as a docking site for SH2 domain of STAT3. STAT3 proteins are then phosphorylated at Tyr705 by JAKs, which mediates interaction between phosphorylated Tyr705 of one STAT3 with the SH2 domain of the other leading to the formation of homodimers. By inducing dimerization of STAT3, Bromberg *et al.* (1999) have shown that spontaneous dimerization leads to constitutively active STAT3 independent of Tyr705 phosphorylation (Bromberg et al., 1999). Dimerized STAT3 then binds to nuclear import proteins, importin-alpha3 and importin-alpha6 and is translocated to the nucleus. Shuttling of STAT3 between nucleus and cytoplasm occurs constantly independent of Tyr705 phosphorylation. Phosphorylation of Ser727 on

STAT3 has also been shown to be important for transactivation of STAT3, although the mechanism still remains unknown (Zhang et al., 1995) (Wen et al., 1995).

Three protein families negatively regulate the JAK/STAT pathway: Phosphatases; PIAS (protein inhibitor of activated STAT); and SOCS3 (Heinrich et al., 2003). It is not known whether the former two are involved in the LIF/STAT3 pathway. However, the latter one is upregulated upon LIF stimulation and acts by inhibiting JAKs by inserting their inhibitory region into the activation domain of JAKs (Boyle et al., 2009).

STAT3 is an essential component for self-renewal and pluripotency of mESCs in the presence of FBS. Inhibition of STAT3 through use of dominant negative mutant of STAT3 prevented its activation resulting in differentiation of mESCs (Niwa et al., 1998). Furthermore, evidence to prove its sufficiency in maintenance of pluripotency, Matsuda *et al.* used a chimaeric STAT3-estrogen receptor (STAT3ER) which induced phosphorylation of Tyr705 upon addition of 4-hydroxytamoxifen (4-OHT), leading to self-renewal of mESCs in presence of FBS without either LIF or feeder cells (Matsuda et al., 1999). Another study confirming the importance of STAT3 in mESCs was carried out by Cinelli *et al.* (2008), where germline competent ESCs from non permissive FVB/N mouse strains using STAT3ER inducible system were generated in absence of LIF upon addition of 4-OHT (Cinelli et al., 2008).

Although, the importance of LIF and STAT3 in self-renewal of mESCs is thouroughly documented, the downstream target genes of STAT3 have remained elusive. Two different ways applied to understand how STAT3 regulates pluripotency include identification of target genes of STAT3 and analysis of protein interactions with STAT3. Various research groups have employed genome-wide approaches like ChIP (chromatin immunoprecipitation) sequencing, ChIP on chip and DNA microarray. One of the microarray analysis study done on STAT3ER ESCs identified a group of 26 STAT3 target genes. The expression of the four of the upregulated genes was restricted to the ICM of the blastocyst. Overexpression of two of these genes, *Pem/Rhox5* and *Pramel7* respectively, in absence of LIF, was shown to be sufficient to maintain the expression of pluripotent ESCs markers (Cinelli et al., 2008). Recently, Casanova *et al.* (2011) reported that *Pramel7* mediated LIF/STAT3 dependent self-renewal in mESCs and ablation of the former induced differentiation of mESCs (Casanova et al., 2011). Cartwright and colleagues have also shown a role for the transcription factor c-myc in self-renewal by functioning as a downstream STAT3 target. It is rapidly downregulated following LIF withdrawal. Overexpression of c-myc can retain cells in an undifferentiated state even in

the absence of LIF where as expression of dominant negative form of it promotes differentiation (Cartwright et al., 2005). The transcription factor Klf4 (Kruppel type zinc finger 4) is another downstream target of STAT3, which maintains mESCs in an undifferentiated state and overexpression of which upregulates the expression of Oct4 and self-renewal of mESCs (Li et al., 2005). Together, these data shed some light on the mechanism involved in maintenance of self-renewal through JAK/STAT3 pathway.

7.1.2. LIF/PI3K/AKT signalling

PI3K pathway is important for cell proliferation, survival and self-renewal of ESCs. Upon binding of LIF, gp130 receptor undergoes phosphorylation of Tyr757, which promotes recruitment of SHP2 to the receptor. The SHP2 is then phosphorylated in a JAK dependent manner and associates itself with the scaffold protein GAB1 (GRB2-associated binder protein), which then recruits PI3K to the cell membrane. PI3K is then activated via phosphorylation of its regulatory subunit p85 and leads to generation of PIP2 (phosphatidylinositol-3, 4-biphosphate) and PIP3 (phosphatidylinositol-3,4,5-trisphosphate). PIP2 and PIP3 are ligands for the PH domains of various signal transducers including serine/threonine kinases, PDK1 (phosphoinositide-dependent kinase 1) and PKB/AKT (protein kinase B). AKT binds to PIP3 and is translocated to the inner cell membrane where it is phosphorylated and activated by PDK1 (Burdon et al., 2002). AKT inhibits GSK3β (glycogen synthase kinases 3b), one of the major proteins, by two different mechanisms. Firstly, it directly inhibits GSK3β activity by phosphorylation of Ser9 and secondly, facilitates nuclear export of GSK3β independently of its phosphorylation, thus blocking its action on target proteins in the nucleus (Doble and Woodgett, 2003) (Bechard and Dalton, 2009). GSK3β promotes ubiquitination and degradation of c-myc by phosphorylation of Thr58 (Bechard and Dalton, 2009) (Cartwright et al., 2005). It also phosphorylates and activates p53, which promotes differentiation of mESCs by suppressing Nanog expression (Lin et al., 2005) (Storm et al., 2007). Thus, inhibition of GSK3β results in an increase of c-myc and Nanog expression both of which play an important role in self-renewal of mESCs. GSK3β also plays a pivotal role in self-renewal of mESCs via Wnt pathway discussed later in detail. Although, LIF/PI3K/AKT pathway and Wnt canonical pathway have GSK3β in common, these pathways work independent of each other. Treatment of mESCs with LIF or enhanced activation of AKT does not increase the levels of b-catenin.

The LIF/PI3K/AKT pathway also plays a role in acetylation of lysine residues on STAT3 (Ohbayashi et al., 2007). Acetylated STAT3 forms more stable dimers and promotes the transcription of target genes without phosphorylation of Tyr705 (Braunstein et al., 2003). However, this has not been shown in ESCs.

The LIF/PI3K/AKT pathway has been associated with ESCs propagation through studies carried out on PTEN, which is a lipid phosphatase that acts as a negative regulator of PI3K pathway. It removes the phosphate from the 3' position of 3-phosphoinositides. PTEN knockout ESCs have been shown to possess enhanced cell viability and increased rate of cell proliferation. These attributes are correlated with the elevated amounts of PIP3 enhanced phosphorylation of PKB and inhibition of pro-apoptotic protein Bad (Sun et al., 1999). It has also been shown by Paling *et al.* (2004) that inhibiton of PI3K with both a reversible inhibitor and a dominant negative p85 subunit resulted in decrease of phosphorylated AKT, GSK3β and ribosomal S6 proteins, increase in ERK1/2 phosphorylation and decline in the ability of the mESCs to self-renew. PI3K inhibition has no influence on the LIF/STAT3 pathway (Paling et al., 2004). Inhibition of MEK reverses the effects of PI3K inhibition suggesting that PI3K pathway maintains self-renewal by blocking ERK1/2 pathway discussed below. Watanabe *et al.* (2006) showed that AKT activation, induced by transfecting constitutively active AKT gene, is sufficient for self-renewal of mESCs in the absence of LIF and feeders (Watanabe et al., 2006). Thus, the studies above indicate a significant role of LIF/PI3K/AKT pathway in self-renewal of mESCs.

7.1.3. LIF/SHP2/MAPK signalling

The third LIF signalling pathway is less well characterized and is not directly involved in the maintenance of self-renewal in ESCs. It regulates various different cellular responses and has a well-documented function in proliferation and differentiation.

Binding of LIF to the LIF receptor induces phosphorylation of gp130 via JAK as mentioned above. This leads to the recruitment and phosphorylation of SHP2 by JAKs. SHP2 then binds to Grb2 (Growth factor receptor bound protein 2)-SOS (son of sevenless) complex, which promotes activation of Ras. SHP2 also associates itself with Gab1 as mentioned above, which stabilizes the SHP2-Gab1-Grb2 complex and enhances the effect of Ras This activation sets off a cascade of transphosphorylation involving Raf and MAPK kinase (MEK) kinases that together contributes to activation of ERK (Extracellular signal related kinases). Activated ERK can then phosphorylate

cytoplasmic targets and also be translocated to the nucleus where it modulates the activity of various transcriptional factors such as c-Jun, c-Fos, Ets and Elk (Kolch, 2000). This pathway is known to induce differentiation of mESCs by downregulating *Nanog* and *Tbx3* (Niwa et al., 2009) (Hamazaki et al., 2006). The Ras/Raf/MEK/ERK cascade can also induce differentiation of mESCs via activation by FGF4. Inhibition of ERK pathway either by use of small molecules to block MEK activity or by forced expression of Erk phosphatases enhances self-renewal of mESCs by reducing differentiation (Burdon et al., 1999b). Inhibition of ERK enhances the activity of STAT3 but it is not known whether the effect is direct or indirect. Thus, the balance between the two pathways converging downstream of LIF determines the choice between self-renewal and differentiation (Burdon et al., 1999a). In mESCs, deletion of SHP2 binding site from chimaeric gp130 receptor blocks its association with Ras and promotes self-renewal response (Burdon et al., 1999b). Genetic disruption of Grb2 results in impaired differentiation, which can be rescued either by introducing Grb2-SOS chimaera or an active form of Ras into the Grb2 knockout ESCs (Cheng et al., 1998). Taken together, these results suggest that the LIF/SHP2/MAPK pathway promotes differentiation of mESCs and suppresses self-renewal response.

7.2. Wnt signalling pathway

Wnt signalling represents one of the key pathways involved in multiple developmental events during the entire lifespan of an organism. It contributes to the establishment of body axis and organogenesis during embryo development and in adult, it plays indispensable roles in tissue homeostasis, cell renewal and regeneration. Historically, Wnt signalling is categorized into two types- canonical and non-canonical pathway according to their dependence on β-catenin. The canonical pathway revolves around stabilization and translocation of β-catenin into the nucleus, which triggers transcription of various Wnt target genes. Non-canonical pathway, on the other hand, involves all Wnt-activated signalling pathways that do not promote β-catenin dependent signalling and includes the planar cell polarity pathway that controls structuring of cytoskeleton and the Wnt/Ca pathway that plays a role in cell adhesion, migration and tissue separation (Tada et al., 2002) (Kühl et al., 2000). Wnt canonical pathway has been shown to play a role in self-renewal of ESCs. However, this function of Wnt signalling is relatively complex and controversial as Wnt signalling mainly contributes to

organogenesis and differentiation of ESCs. I will focus on Wnt canonical pathway and its role in maintaining pluripotency of ESCs below.

In the absence of Wnt signal, the destruction complex regulates the stability of b-catenin, which is a key component of the signalling output of Wnt canonical pathway. The multiprotein destruction complex consists of the scaffold proteins Axin and APC (Adenoma Polyposis Coli) and two constitutively active serine threonine kinases GSK3β and CK-1 (Casein kinase 1). When Fz (Frizzled)/LRP (low density lipoprotein receptor related proteins) receptor are not bound by Wnt, GSK3β and CK1 phosphorylate Axin bound β-catenin at its ser/thr residues. This complex is then recognized by the F-box WD repeat protein b-TrCP, a component of an E3 ubiquitin ligase complex where β-catenin undergoes ubiquitination and rapid degradation by the proteasome (Aberle et al., 1997), thus blocking β-catenin dependent activation of target genes in the nucleus. Upon activation of receptors by Wnt ligands, Dvl (Dishevelled) is recruited to the the receptor complex where it subsequently multimerises and induces formation of LRP associated Wnt signalosomes, which recruits Axin to the phosphorylated LRP receptor along with the kinases, thus destablilising β-catenin degradation complex (Schwarz-Romond et al., 2007) (Bilic et al., 2007). Recent studies by Li *et al* (2012), show that activation of Wnt signal leads to inhibition of β-catenin ubiquitination by E3 Ubiquitin ligase complex. The complex becomes saturated by phosphorylated form of β-catenin and the newly synthesized β-catenin thus accumulates in the cytoplasm and eventually translocates to the nucleus to activate Wnt target genes (Li et al., 2012). The mechanism behind the shuttling of β-catenin between the cytoplasm and nucleus is still not clear, although the recent data suggests a function for microtubules in its nuclear import (Sugioka et al., 2011). β-catenin also plays another role, independent of its signalling function, in the cell adhesion system where it binds to the intracellular domain of E-cadherins (Peifer et al., 1992).

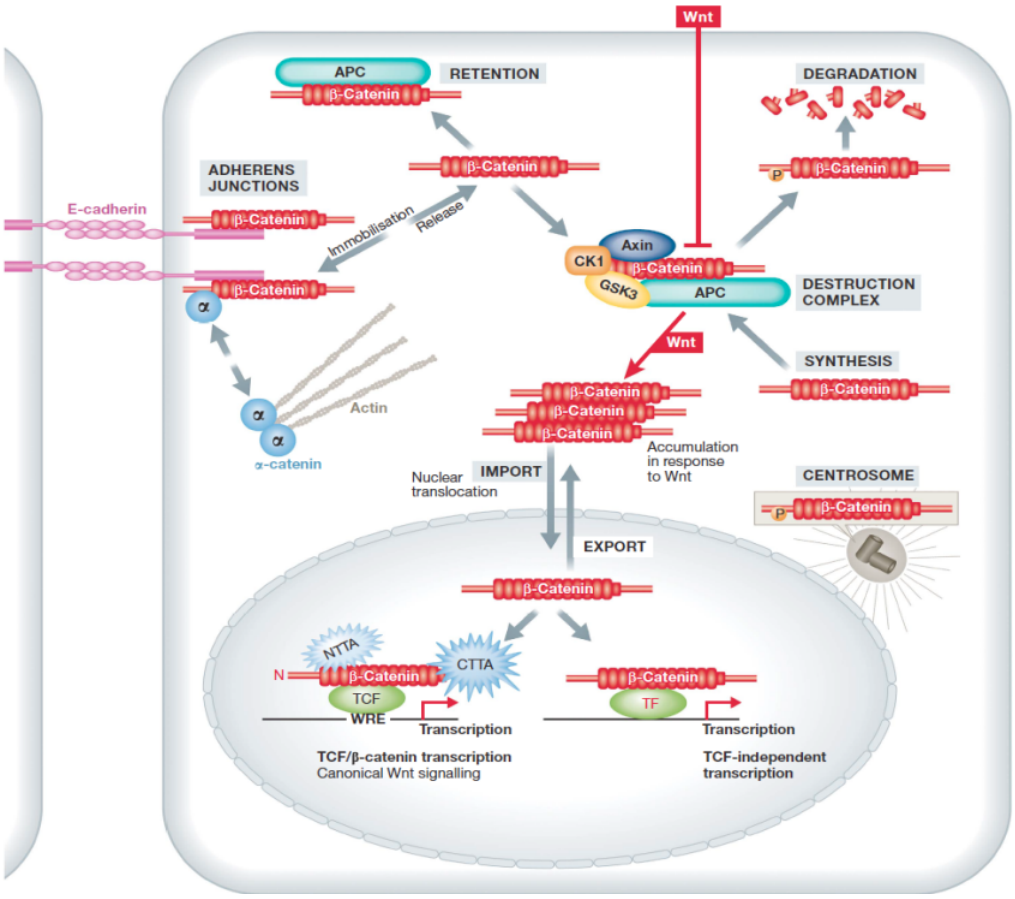

Figure 7: Different faces of β -catenin

B-catenin plays a dual role in the cell. At the adherens junctions, it binds to E-cadherin and a-catenin and modulates the actin cytoskeleton. Free b-catenin, which is not involved in cell adhesion system is phosphorylated by degradation complex and degraded. In the presence of Wnt signalling, activity of destruction complex is inhibited and β-catenin degradation is blocked. Free β-catenin is then translocated to the nucleus where it binds to TCF/LEF family of transcription factors and others to initiate transcription of Wnt target genes. Amount of β-catenin in the nucleus is modulated by its nuclear import/export. CTTA- C terminal transcriptional activators; NTTA- N-terminal transcriptional activators. Taken from (Valenta et al., 2012)

In the nucleus, β-catenin can activate transcription of its target genes by binding to DNA binding partners, which bring it to the promoters of the target genes. As β-catenin does not possess DNA binding domain, it can initiate transcription only by being a member of multimeric complexes (Huber et al., 1997) (Xing et al., 2008). In the Wnt off state, TCFs act as transcriptional repressors by binding to Groucho/TLE (transducing-like enhancer of split) transcriptional repressors preventing gene transcription (Cavallo et al., 1998) (Roose et al., 1998). In the Wnt on state, binding of β-catenin physically replaces Groucho/TLE and converts TCFs into transcriptional activator of Wnt target genes (Daniels and Weis, 2005). β-catenin acts as the central transcriptional activator of Wnt target genes. TCF (T-cell factor)/Lef (Lymphoid enhancer factor) transcription factors act as the main nuclear partners of b-catenin.

Mammals possess four TCF genes: TCF1, TCF3, TCF4 and LEF1 amongst which TCF3 is the most abundant in ESCs. TCF3 has been shown to colocalise with core-pluripotency associated factors Oct4, Sox2 and Nanog including their own promoters. This study also showed that knockdown of *TCF3* in mESCs lead to upregulation of Oct4, Sox2 and Nanog suggesting that TCF3 acts as a transcriptional repressor (Cole et al., 2008). Independently, Pereira *et al.* (2006) also demonstrated inhibition of Nanog by TCF3 (Pereira et al., 2006). Recently, Smith and colleagues extended these findings by showing that inhibition of GSK3β induces interaction of β-catenin with TCF3, which inhibits its repressive effect on the activity of core pluripotency factors (Wray et al., 2011). This is consistent with the findings from two studies where *TCF3* null ESCs are pluripotent in the absence of LIF, replace the requirement of GSK3β inhibitor in defined conditions and overexpression of it destabilizes self-renewal even in the presence of LIF and serum (Wray et al., 2011) (Yi et al., 2008). Recently, Smith and colleagues identified *Esrrb* as a direct functional target of TCF3 downstream of GSK3β inhibition. It is both essential and sufficient for the response to GSK3β inhibition. Knockdown of Esrrb even in the presence of GSK3β inhibitor led to the loss of pluripotency markers. Conversely, overexpression of it phenocopied GSK3β inhibition or TCF3 deletion by blocking differentiation of ESCs (Martello et al., 2012). However, Merrill and colleagues revealed that this cannot be the only mode of action. They showed that β-catenin inhibits TCF3 mediated repression and activates target genes together with TCF1 (Yi et al., 2011). Thus, TCF3 and TCF1 combination contributes to Wnt stimulated self-renewal. Albeit TCF/Lef dependent β-catenin mediated transcription, β-catenin has also been shown to physically interact and form a complex with Oct4 and promotes the activity of the latter to regulate pluripotency (Kelly et al., 2011).

ten Berge *et al.* have recently reported the physiological role of canonical Wnt pathway. They showed that Wnt3a protein secreted by mESCs in combination with LIF can support the expansion of mESCs. Inhibition of paracrine or autocrine activity of Wnt by blocking Wnt secretion or Wnt neutralization after secretion results in the transition of mESCs to mEpiSCs. Their demonstration that Wnt3a can replace GSK3β inhibitor in 2i condition indicated that inhibition of GSK3β is attributed to its ability to activate Wnt pathway probably through the role of TCF3 in regulating core pluripotency genes (ten Berge et al., 2011). However, there is no clear-cut evidence to prove that Wnt canonical pathway alone is sufficient to maintain long-term self-renewal of mESCs.

In addition to this, impact of deletion of β-catenin in mESCs still remains a controversy. Lyashenko *et al.* reported that β-catenin null mESCs remain pluripotent whereas earlier studies suggested that ablation of β-catenin promotes transition to EpiSCs (Lyashenko et al., 2011) (Anton et al., 2007) (Soncin et al., 2009). These discrepancies in the phenotype can be due to the different genetic background of these mESCs lines. Indeed, Hanna *et al.* found that the stability of naïve pluripotent state in LIF and serum condition depends on the genetic background (Hanna et al., 2009). Thus, further studies need to be carried out to provide an important insight into the role of Wnt signalling in maintenance of pluripotency and self-renewal.

8. Gene of interest: Pramel7

In mouse, *Pramel7* is present on chromosome 2D and consists of conserved leucine rich repeats essential for protein-protein interactions and lacks conserved domains typical for transcription factors thus does not directly play a role in gene transcription regulation. Two studies carried out by Cinelli and colleagues demonstrate *Pramel7* as a gene involved in maintenance of pluripotency in mESCs (Cinelli et al., 2008) (Casanova et al., 2011). It was initially found in a microarray study carried out by Cinelli *et al*. (2008) that *Pramel7* was strongly upregulated in conditionally overexpressing Stat3 ESCs (Cinelli et al., 2008). *In vivo* analysis of wild type pre-implantation embryos revealed expression of Pramel7 in central part of the morula and the ICM of the blastocyst. Its expression was absent in the post-implantation stage embryos indicating a possible role in maintenance of pluripotency *in vivo*. *In vitro*, ESCs overexpressing Pramel7 can be propagated for several passages even in the absence of LIF. It has also been shown that overexpression of Pramel7 prevents ESCs differentiation by promoting gradual reduction of Erk phosphorylation. Despite the presence of LIF and overexpression of STAT3 in ESCs, knockdown of *Pramel7* induces differentiation of ESCs. Henceforth, it can be said that LIF mediated self-renewal is dependent on Pramel7 expression. However, unlike WT ESCs, LIF induction on Stat3 null ESCs did not upregulate *Pramel7* transcription confirming that Pramel7 is a direct downstream target of LIF/Stat3 pathway. Interestingly, the basal level of *Pramel7* in Stat3 null ESCs is higher than in WT ESCs suggesting a parallel pathway controlling *Pramel7* transcription. In presence of 2i, upregulation of *Pramel7* upon LIF induction was significantly delayed. Intriguingly, presence of CH, an inhibitor of GSK3β, impaired the transcription of Pramel7 in WT ESCs (Casanova et al., 2011). Taken together, these results suggest that the combined activity of LIF/STAT3 pathway and one of the GSK3β pathways control *Pramel7* transcriptional regulation.

9. Goal of the thesis

The overall aim of the thesis is to gain insight into different mechanisms regulating pluripotency in mouse and rat embryonic stem cells. The present thesis comprises of two projects:

- Project I: Establishment of pluripotent germline competent rat embryonic stem cells from Brown Norway strain
- Project II: Elucidating the molecular mechanisms regulating transcription of Pramel7 in mouse embryonic stem cells

Project I: Establishment of pluripotent germline competent rat embryonic stem cells from Brown Norway strain

Goal of this project: To establish authentic rat embryonic stem cells which are pluripotent and germline-competent. For this purpose, the following experiments were designed:

- Breeding of Brown Norway rats
- Collection of embryos
- Derivation and expansion of embryonic stem cells
- *In vitro* characterisation for pluripotency of the established cell lines
- *In vivo* characterisation for pluripotency of the established cell lines
- Generation of chimera and germline transmission

However, as this project was not completely successful, it was necessary to further understand the molecular mechanisms regulating pluripotency in mouse ESCs and then transfer the knowledge to rat embryonic stem cells. Therefore, a decision was made to focus on a parallel project related to maintenance of pluripotency in mouse embryonic stem cells described below.

Project II: Elucidating the molecular mechanisms regulating transcription of Pramel7 in mouse embryonic stem cells

Goal of this project: To deeper understand the molecular mechanisms driving the transcription of *Pramel7* by studying different pathways involved in self-renewal and differentiation of the mouse embryonic stem cells.

In particular, investigation was carried out into the link between *Pramel7* repression and inhibition of GSK3β by addressing the following questions.

- How is the expression of *Pramel7* regulated by the GSK3β downstream effector, β-catenin via Wnt/canonical pathway?
- Is the transcriptional control of *Pramel7* occuring directly via GSK3β?
- Is it possible that regulation of *Pramel7* occurs via other GSK3β pathways such as MAPK/ERK pathway?

To examine the link between Pramel7 and the GSK3β target, β-catenin, following experiments have been designed.

- Derivation and characterisation of different β-catenin mutants: knockout, N-terminal, C-terminal and constitutively active S33Y β-catenin mutant
- Analysis of *Pramel7* transcription in the above mutants
- Examine STAT3 phosphorylation in β-catenin KO ESCs

To investigate the direct role of GSK3β in driving transcription of *Pramel7,* below given experiments have been performed.

- Carry out gene expression analysis to elucidate the transcription of Pramel7 via GSK3β using three different GSK3β mutants: GSK3α/β double knockout (DKO), kinase inactive GSK3β; DKO_K85A and wild type GSK3β; DKO_GSK3β
- Determine if there is any physical interaction between GSK3β, Pramel7 and STAT3

To explore the possibility of the involvement of MAPK/ERK pathway in regulation of Pramel7, following experiments have been planned.

- Analysis of *Pramel7* expression in WT ESCs in both presence and absence of PD, a specific inhibitor of Erk kinase
- Elucidate the regulation of *Pramel7* in GSK3β mutants in presence of Erk inhibition

The above experiments have shed some light on the molecular mechanisms involved in the regulation of *Pramel7* at the transcriptional level.

B. Results

1. Project I: Establishment of pluripotent germline competent rat embryonic stem cells from Brown Norway strain

1.1. Derivation of rat ESC lines from Brown Norway (BN) rats

The efficiency of isolating BN rat ESCs is shown in the table below.

Table 3: Derivation of rat ESCs in 2i+LIF

	No of ICMs plated	Outgrowths	Continuous ESC lines
Round 1	5	5	0
Round 2	4	3	0
Round 3	6	4	0
Round 4	8	8	0
Round 5	5	5	3
Round 6	4	2	2

Outgrowth refers to the adherent primary explants. Continuous refers to indefinitely proliferating cultures of undifferentiated cells.

Six independent experiments were carried out where immunosurgery on the E4.5dpc embryos was successful and showed outgrowths in all the experiments. However, only five ESC lines were established from these six independent rounds because a high number of outgrowths failed to form ESC colonies on subsequent plating. Derivation protocol was optimized in the different rounds to successfully establish the ESCs. Initially, classic B6 feeders were plated on gelatinised plates and trypsin was used for dissociation of outgrowths. However, it was not possible to establish any ESC lines with this strategy and the protocol that led to the successful establishment of BN ESC lines comprised of plating SNL feeders over laminin coated plates and using Accutase for dissociation of outgrowths.

All lines showed similar morphology and growth characteristics except BN_6.1 and BN_6.2, which proliferated slightly slower compared to the others. All ESC lines formed compact colonies similar to mESCs and the individual cells showed a high nucleus to cytoplasm ratio, a typical characteristic of ESCs (Fig. 8). Large colonies had the tendency to detach from the feeder layer and were therefore passaged every 2-3 days. All the established ESC lines could be propagated continuously for more than a year with no discernible change in proliferation rate and morphology.

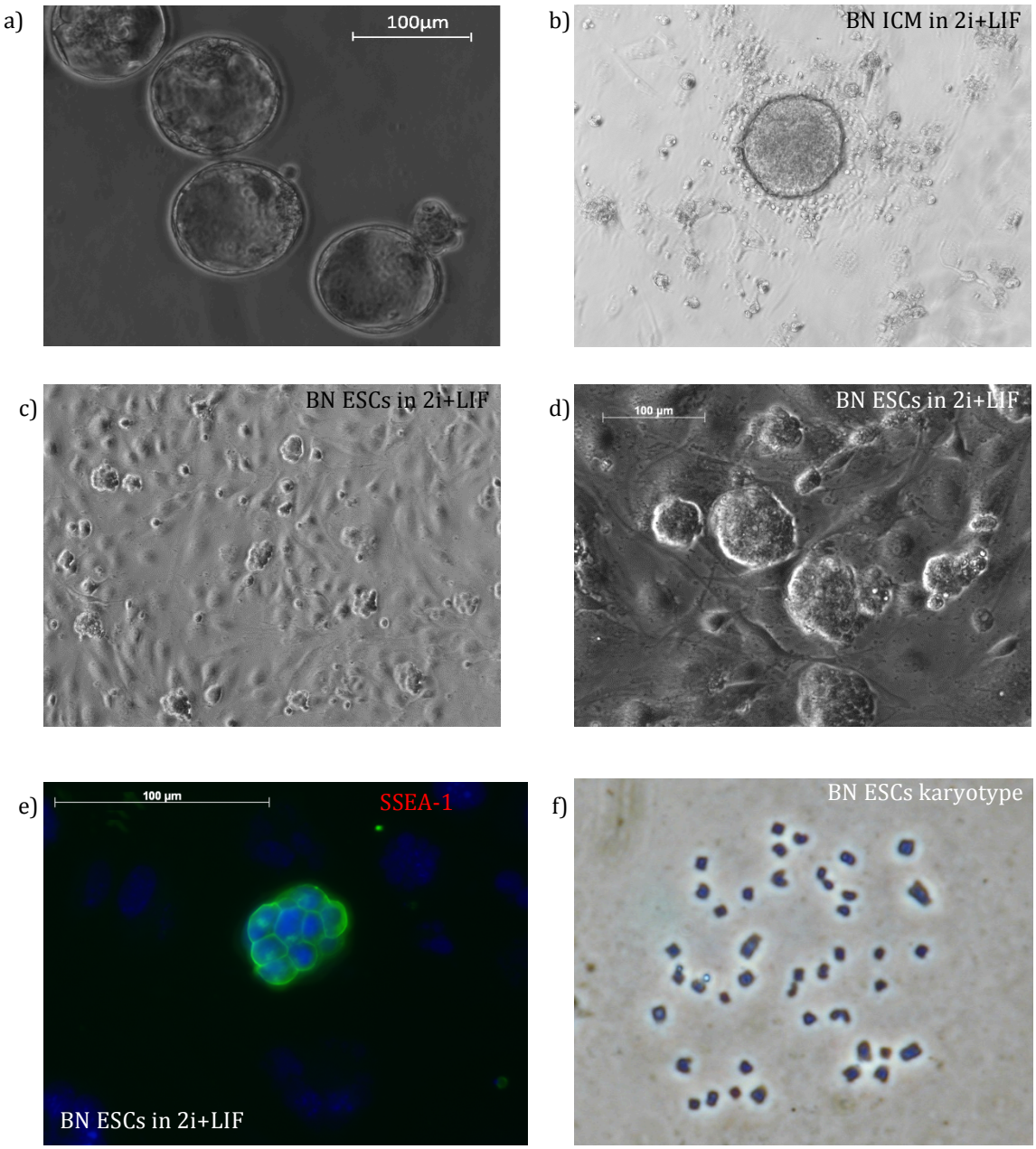

Figure 8: Establishment of rat ESCs

a) Late blastocyst stage embryos used for derivation of rat ESCs b) Inner cell mass plated on feeders after immunosurgery c)&d) Morphology of 2i+LIF colonies on feeders at low and high magnification e) typical ESCs characteristic, showing high nucleus to cytoplasm ratio f) Normal karyotype, 42 XY shown by Giemsa staining

Karyotype analysis indicated that all the ESC lines had 70% normal karyotype as shown in the table below.

Table 4: Karyotype analysis for established rat ESC lines

ESC line	No. of chromosome spreads	No of Normal karyotype
BN_5.1	39	30
BN_5.3	27	17
BN_5.5	50	33
BN_6.1	27	22
BN_6.2	22	15

1.2. Characterization of the ESC lines

To assess the identity of the established ESC lines, expression of pluripotent markers was examined. Nuclear localized Oct4 and cell surface SSEA-1 protein were detected by immunofluorescence microscopy. Strong positive staining was observed for alkaline phosphatase in the ESC lines confirming their pluripotent state (Fig. 9).

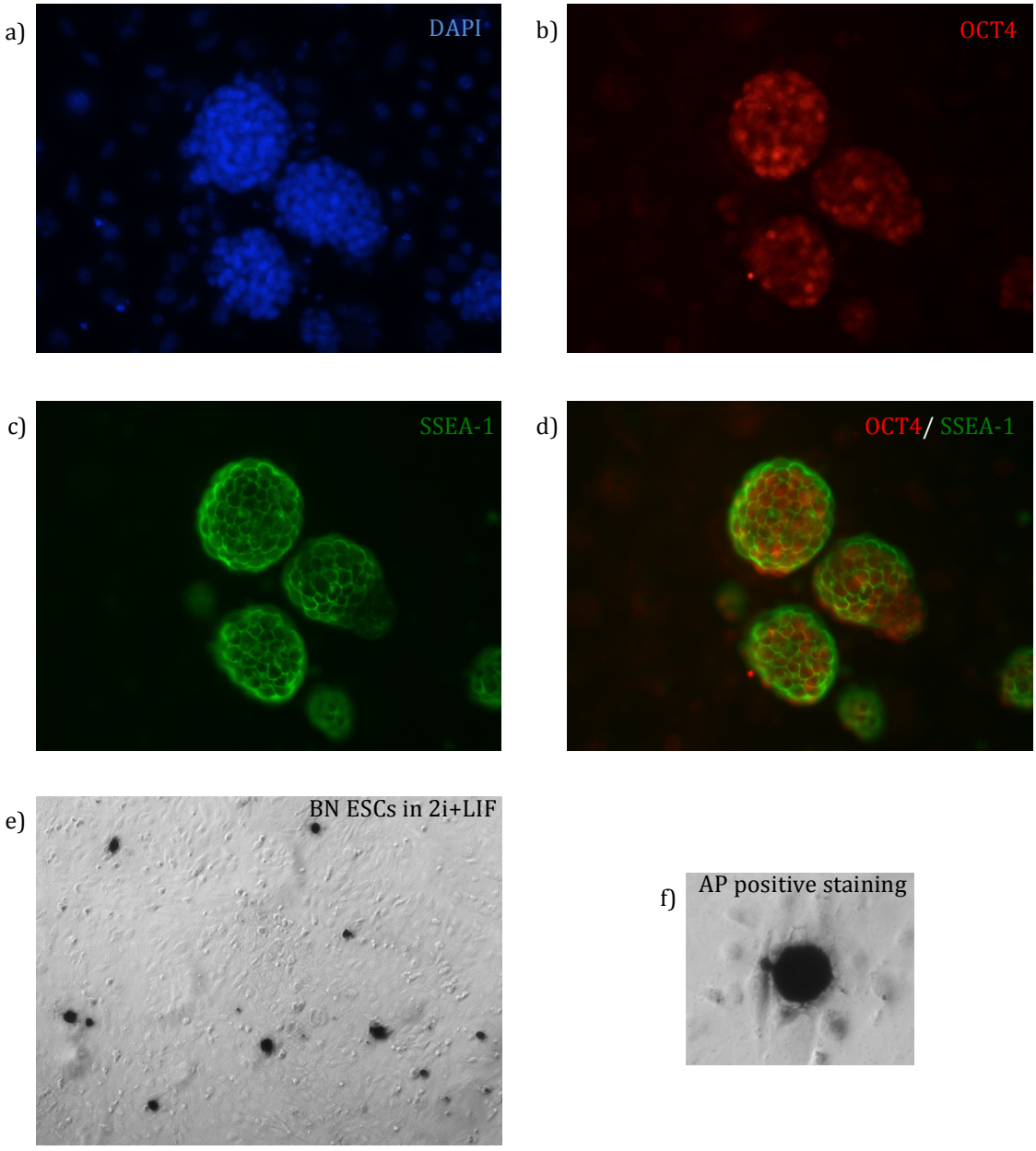

Figure 9: Expression of pluripotency markers in rat ESCs lines

a-d) Immunofluorescence staining of Oct4 and SSEA-1 in 2i+LIF established ESC line e)&f) Typical Alkaline phosphatase positive staining showing undifferentiated core with differentiated cells on the boundary in low and high magnification

The differentiation potential of ESCs was tested using protocols to form embryoid bodies and to differentiate ESCs *in vitro* into neurons and muscles. They formed compact spherical embryoid bodies like mESCs. After 10 days induction, beating cells were evident inside the embryoid bodies, representative of cardiomyocytes. Cells cultured in unsupplemented N2B27 expressed neural precursor marker and stained positive for β-III TUBULIN and PAX6. On exposure to serum in absence of feeders, 2i and LIF, all ESC lines differentiated and showed positive staining for smooth muscle actin (SMA), a marker for mesoderm (Fig. 10). However, induction of both the neural and smooth muscle differentiation caused a higher cell death in the established ESC lines specifically BN_6.1 and BN_6.2.

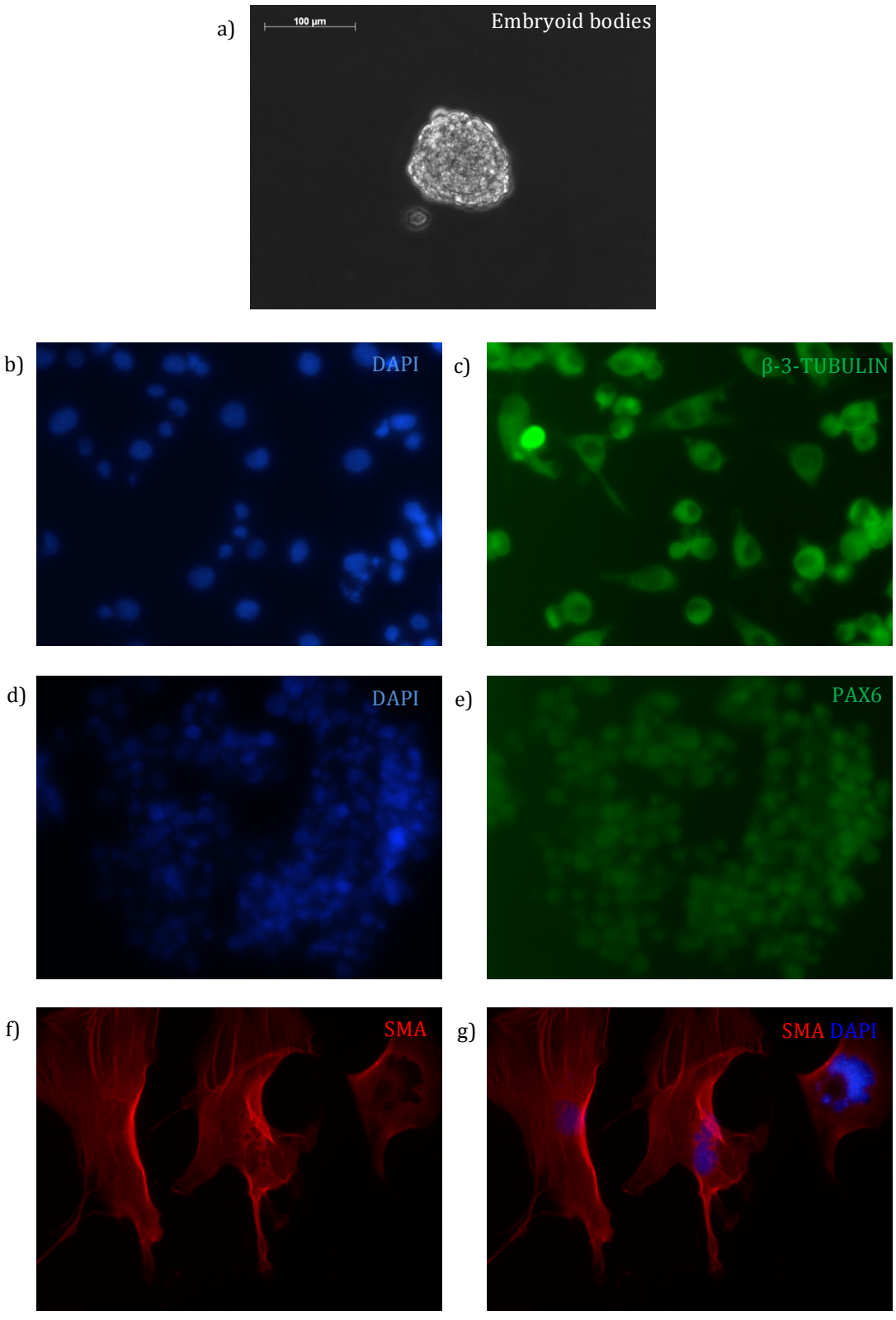

Figure 10: In *vitro* differentiation of rat ESCs

a) Typical compact spherical embryoid body formation from established lines after 8 days in differentiation medium b-e) Immunostaining for β-III tubulin and Pax6 after 10 days of serum-free differentiation on laminin f) & g) Positive staining for SMA after 10 days of differentiation in presence of serum indicating differentiation towards mesodermal lineage

To examine the differentiation potential of rat ESCs *in vivo*, the ESCs were injected subcutaneously into the severe combined immunodeficient (SCID) mice, which have impaired ability to make T- or B- lymphocytes and are immunocompromised. Four of the ESC lines, 5.1, 5.3, 5.5 and 6.2, injected exhibited a macroscopic tissue mass at the site of injection. Immunohistochemical staining of the sections revealed classical features of a teratoma and exhibited positive staining for β-III tubulin, smooth muscle actin and β-catenin, markers of ectoderm, mesoderm and endoderm respectively (Fig. 11). Thus the BN ESC lines are capable of producing teratomas and exhibiting mature multi-lineage differentiation.

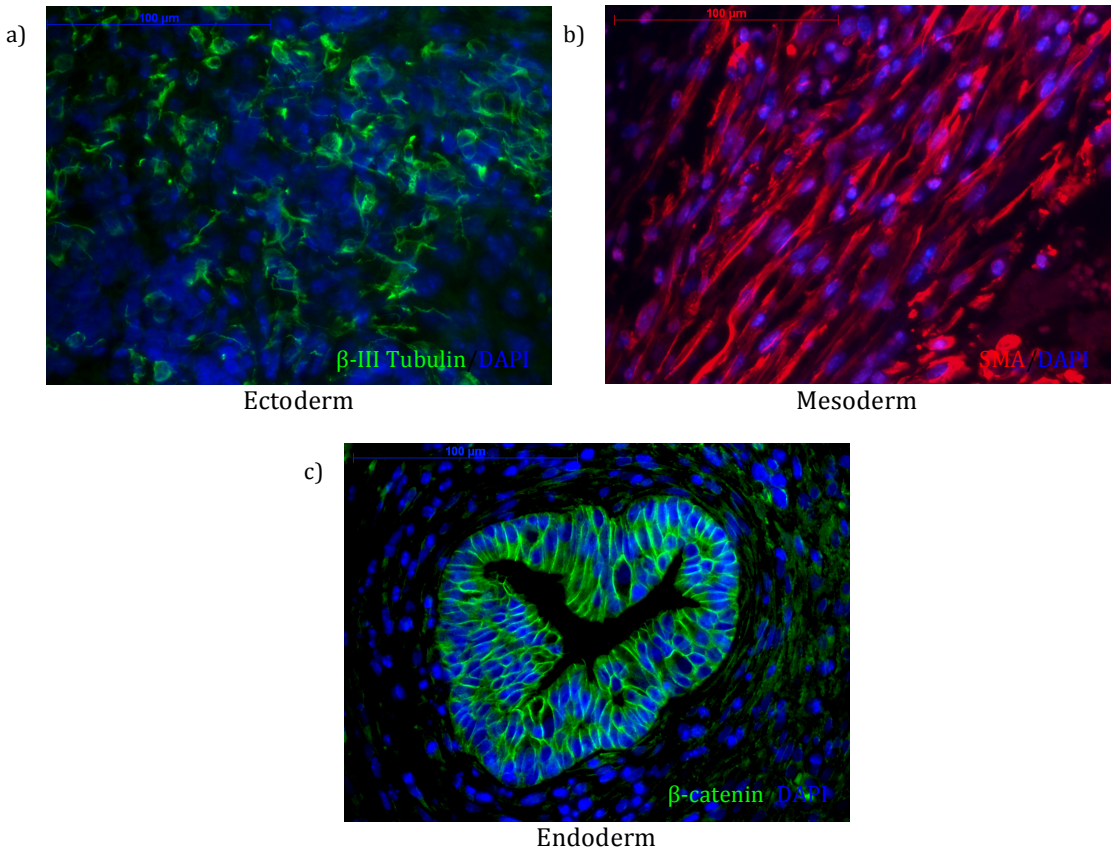

Figure 11: Immunofluorescence staining for the teratoma sections

a) Tumour sections stain positive for β-III tubulin, a well-known neuron specific marker b) Sections also stained positive for smooth muscles, represented by immunofluorescent staining for anti-α smooth muscle actin, SMA c) Colon-like structures stained for β-catenin, an endoderm marker

1.3. Chimera production from rat ESCs

After expansion of ESCs for six passages, an attempt was made to generate chimeras from four of the rat ESC lines. The BN rat ESC lines were injected into Lewis strain blastocysts and then transferred into uterine horns of pseudo-pregnant Wistar females. One live pup was born from BN_6.2G blastocyst injection round. However, it showed no coat color chimerism.

Table 5: Generation of chimeras

Cell line	Passage no	Host embryo	Foster mother	Injected embryos	Live pups	Chimeras
BN_5.1G	7	Lewis	Wistar	30	0	0
BN_5.3G	7	Lewis	Wistar	31	0	0
BN_5.5G	7	Lewis	Wistar	35	0	0
BN_6.2G	7	Lewis	Wistar	12	1	0

2. Project II: Elucidating the molecular mechanisms regulating transcription of *Pramel7* in mouse embryonic stem cells

In the initial study carried out on *Pramel7,* it was shown that the presence of CH, a GSK3β inhibitor, retarded the transcription of *Pramel7* (Casanova et al., 2011). The published study has been attached as an Annex. Thus, it is plausible that at the mRNA level, *Pramel7* is regulated by the parallel activity of both LIF/STAT3 and one of the pathways involving GSK3β.

Based on the above hypothesis, experiments were planned to first examine the link between β-catenin, an important GSK3β downstream effector and *Pramel7.* Follow up studies were carried out to understand the molecular mechanism by which GSK3β or its downstream targets regulate the transcription of *Pramel7.*

2.1. Generation and Characterisation of β-catenin knockout, N-terminal and C-terminal mutant ESCs

β-catenin protein is made up of 12 imperfect Armadillo repeats flanked by N- and C-terminal tails. The N-terminal domain of the first repeat region is essential for binding of Legless/B-cell lymphoma 9 (BCL9) to β-catenin. BCL9 in turn recruits Pygopus which, acts as a transcriptional activator of Wnt target genes. The central region is necessary for TCF/LEF mediated transcription and binding to the components of the adherens junctions. The C-terminal tail along with Arm repeats R11- R12 mediates the interaction with various proteins such as TATA- binding protein (TBP), CREB binding protein (CBP)/p300, Brahma/Brahma-related gene 1 (Brg1), Mediator subunit 12 (MED12) and Hyrax/ Parafibromin. Several of these are involved in chromatin remodeling complexes or along with β-catenin promote the transcription of Wnt target genes. Three mutant variants for signalling and adhesion functions of β-catenin were successfully derived. Given below is a scheme representing different mutations in β-catenin used for the study (Fig. 12). β-cateninKO ESCs do not have the ATG start codon for β-catenin thus no protein is expressed. N-terminal mutation D164A prevents the binding of N-terminal co-activator BCL9 to β-catenin required for recruitment of Pygopus and transcription of Wnt target genes. C-terminal truncation does not allow binding of C-terminal co-activators mentioned above and prevents the transcription of various Wnt target genes. Thus, the last two mutants behave as signalling mutants for β-catenin.

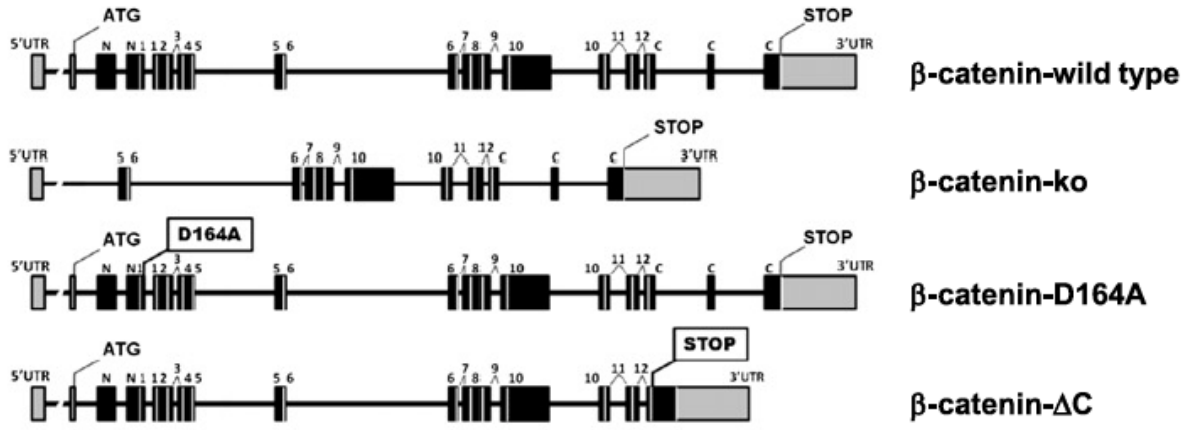

Figure 12: Scheme representing different β-catenin mutants derived

β-catenin protein consists of a central region made of 12 armadillo repeats as shown in the WT figure. β-catenin KO does not possess the ATG start codon thus no protein is formed. β-catenin-D164A is an N-terminal mutant containing a point mutation, which prevents the binding of N-terminal coactivator BCL9 to β-catenin. β-catenin-ΔC mutant carries a C-terminal truncation, which blocks the C-terminal coactivators from binding to β-catenin. Taken from (Valenta et al., 2011)

The mutant ESCs were generated by breeding the heterozygous mice harbouring the β-catenin mutant alleles, obtained from Prof. Basler group. Mouse ESCs were derived and expanded from E2.5 embryos under serum free conditions using 2i+LIF system (Table 6), inhibiting mitogen activated kinase kinase using PD0325901 and GSK3β using CHIR99021(Ying et al., 2008).

Table 6: Derivation of different β-catenin mutant ESCs

Genotype	No. of breeding rounds	No. of ICMs	Outgrowths	Continuous lines
β-catenin KO	4	15	10	4
β-catenin D164A	6	36	5	5
β-catenin Δ C	15	2	2	1

ESC lines were established, genotyped and then chosen for further experiments. They were characterized by analyzing for stem cell markers, formation of embryoid bodies and *in vitro* differentiation of ESCs as shown in the table 7 below.

Table 7: Characterisation of β-catenin ESCs

ESC nomenclature	Genotype	Expression of Pluripotency markers	Embryoid body formation	In vitro differentiation
β-catenin4.1WT	Wild type	X	X	X
β-catenin4.2KO	Knock out	X	X	X
D164A_5.2HM	N-terminal homozygous mutant	X	X	X
D164A_5.5HM	N-terminal homozygous mutant	X	X	X
D164A_5.4WT	Wild type	X	X	X
Δ C1.1_HM	C-terminal homozygous mutant	X	X	X

β-catenin-WT, -D164A and -ΔC ESC lines are pluripotent and express the pluripotency markers and have the ability to form embryoid bodies and differentiate into the three germ layers upon induction with the differentiation stimuli

β-catenin4.2KO (β-cat4.2KO) ESCs line was derived from breeding β-catenin heterozygous mice and was chosen as a knockout line for further experiments. β-cat4.2KO ESCs lacked detectable β-catenin protein. This was confirmed by Western blot (Fig 13a) and PCR analysis. In order to determine the functional consequence on Wnt signalling, quantitative real time PCR (QRT-PCR) was performed on the known TCF target genes, Axin2 and CyclinD1 (Fig. 13b). Both the genes were downregulated in comparison to wild type in 2i+LIF medium. 2i medium contains CH which inhibits GSK3β and blocks degradation of β-catenin. Thus, it promotes the expression of Wnt target genes. β-catKO ESCs do not activate Wnt target genes in 2i conditions confirming absence of β-catenin in these cells.

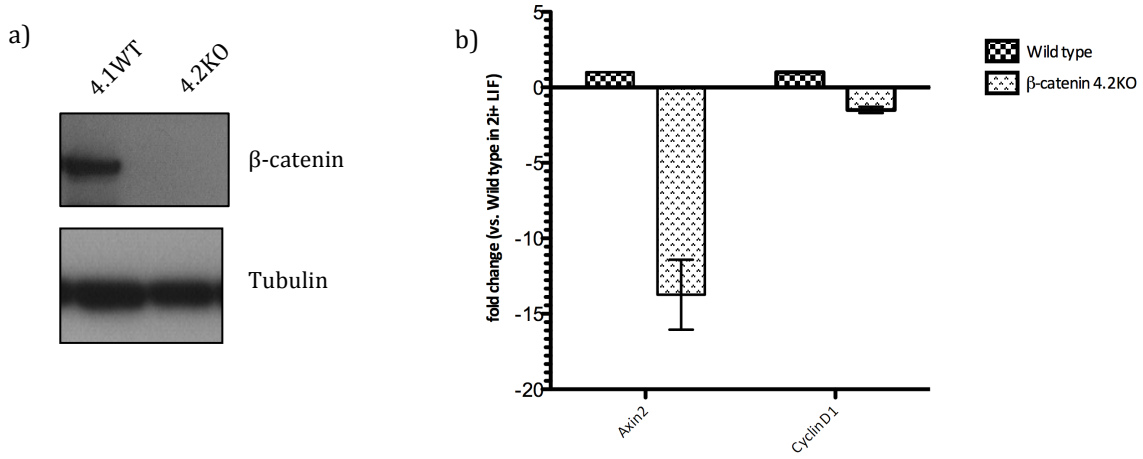

Figure 13: Analysis of β-catKO ESCs

a) Immunoblot showing lack of β-catenin in 4.2KO ESCs b) Histogram showing downregulation of β-catenin target genes, Axin2 and CyclinD1 in β-cat4.2KO ESCs

Unlike wild type ESCs, they have a flattened morphology suggesting cell-adhesion defects caused by the absence of β-catenin. An increased number of single cells observed in culture confirmed cell-cell adhesion abnormality (Fig. 14a). However, expression of self-renewal genes *Oct4, Nanog* and *Rex1* was not altered supporting the findings that cell adhesion is not essential for maintenance of self-renewal (Fig. 14b & 14e) (Ying et al., 2008) (Soncin et al., 2009).

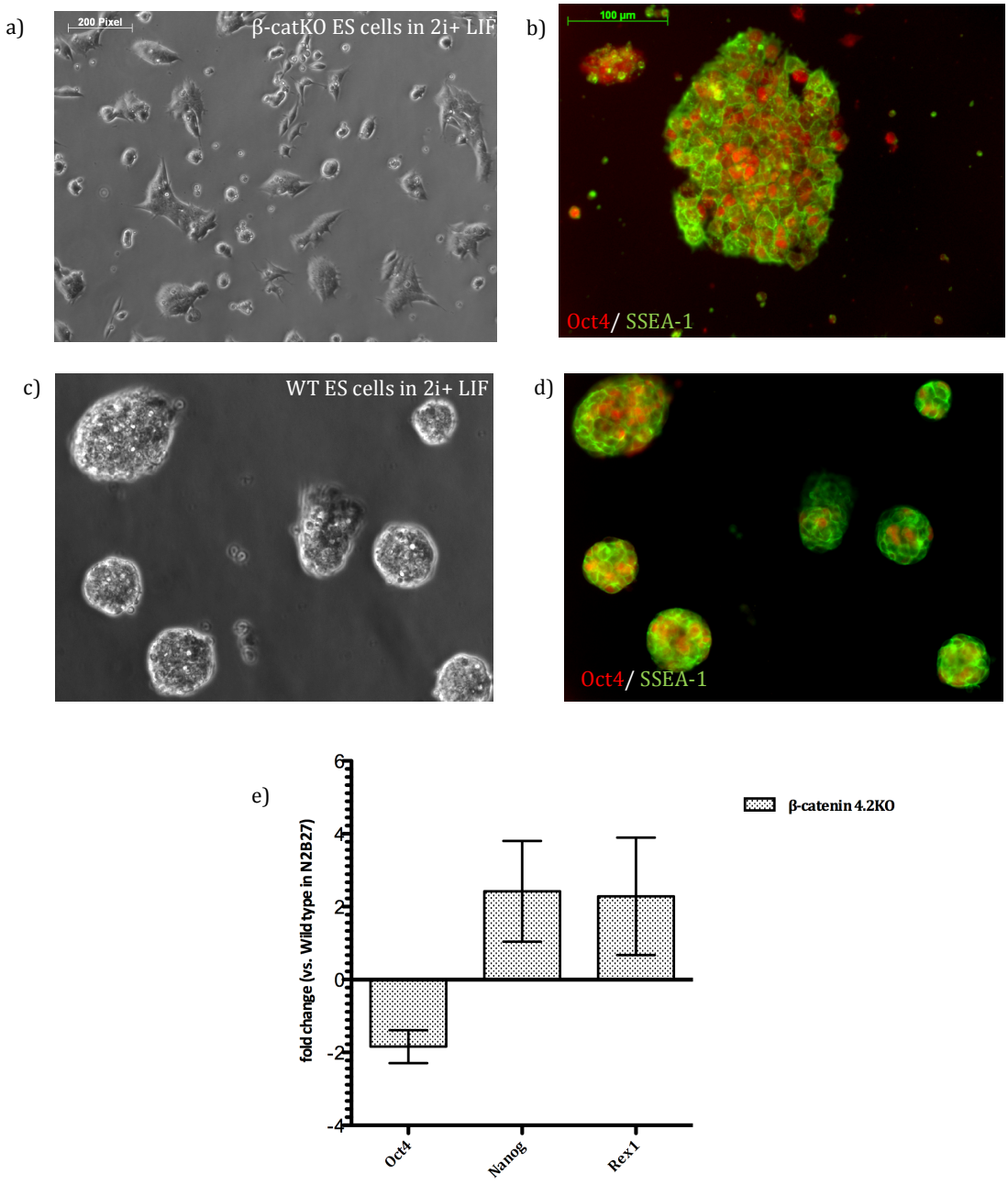

Figure 14: Characterisation of β-cat4.2KO ESCs

a) Phase contrast image of β-cat4.2KO ESCs cultured in 2i+LIF, show flattened morphology for the ESC colonies b) Immunostaining on β-cat4.2KO ESCs shows positive staining for pluripotency markers Oct4 and SSEA-1 c) Phase contrast image of WT ESCs showing round compact morphology d) WT ESCs are positive for nuclear localised Oct4 and cell-surface marker SSEA-1 e) Relative expression of pluripotency markers, *Oct4*, *Nanog* and *Rex1* in comparison to WT ESCs cultured in 2i+LIF

The *in vitro* differentiation potential of these cells was analysed using embryoid body formation and differentiation protocols. Embryoid bodies formed from β-cat4.2KO ESCs did not have a compact ball-like structure, were smaller compared to WT embryoid bodies and started to disaggregate after approximately a week of differentiation (Fig. 15a and 15b). Expression profiles of ectodermal marker genes *FGF5* and *Sox9* in β-cat4.2KO embryoid bodies was almost similar to WT ones. However, expression of endoderm marker gene *Gata4* was slightly reduced and mesoderm marker gene *T-brachyury* was completely absent in β-cat4.2KO embryoid bodies suggesting an abnormality in mesendodermal differentiation (Fig. 15c).

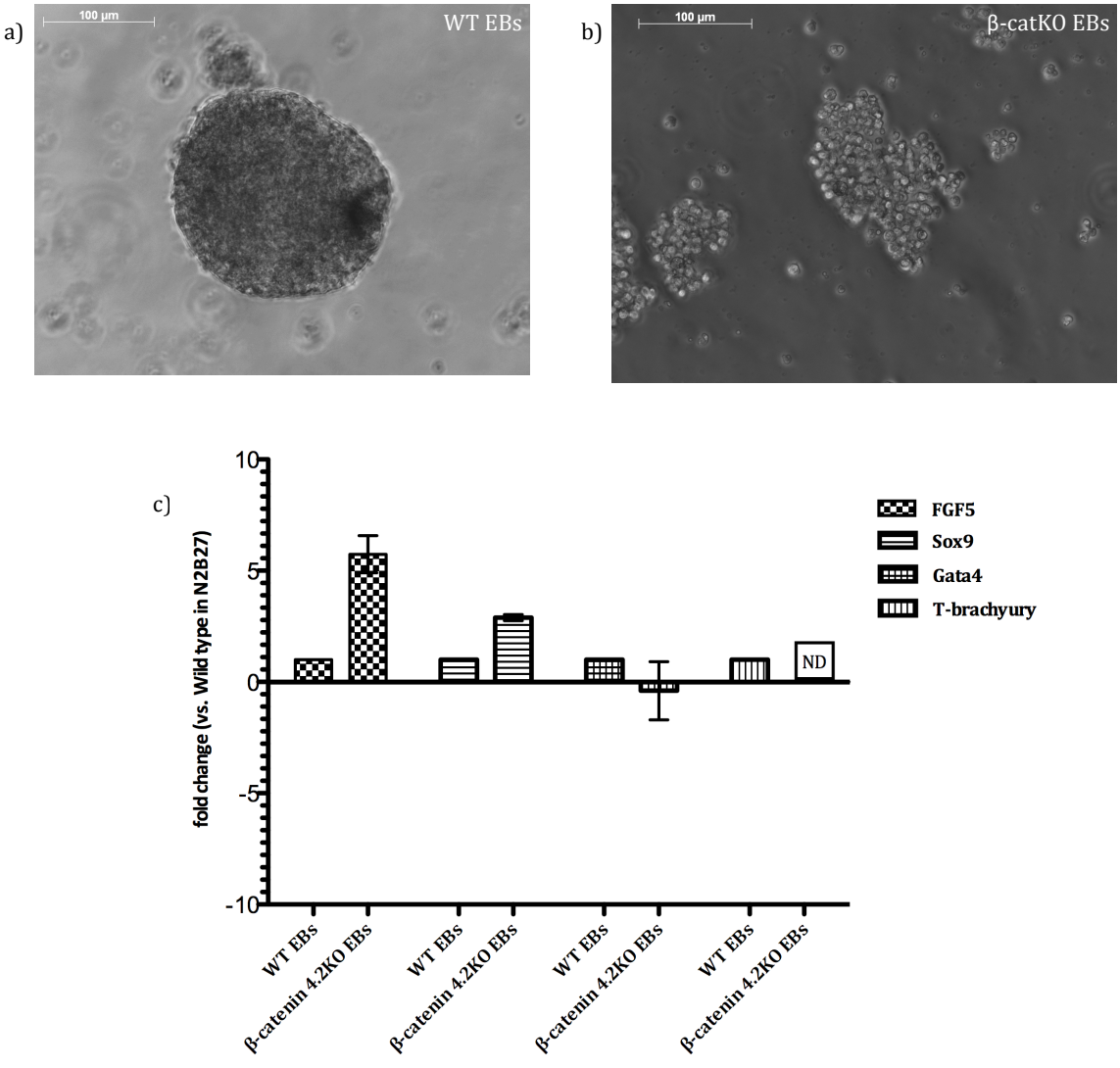

Figure 15: In vitro differentiation of β-cat4.2KO ESCs

a) Spherical compact ball- like WT embryoid bodies b) Phase contrast image showing disaggregating morphology of β-cat4.2KO embryoid bodies and floating single cells confirming cell-cell adhesion abnormalities b) Histogram showing upregulation of ectoderm markers, *FGF5* and *Sox9* and absence of mesoderm marker *T-brachyury*

In contrast to β-catenin4.2KO ESCs, D164A and ΔC homozygous mutant ESCs both have the classical compact round morphology of ESCs like the WT ESCs (Fig. 16a). They express the known pluripotent markers *Oct4* and *Nanog*, although the expression of *Oct4* appears to be downregulated in the mutants in comparison to WT ESCs as seen in the real time analysis (Fig. 16c & 16d).

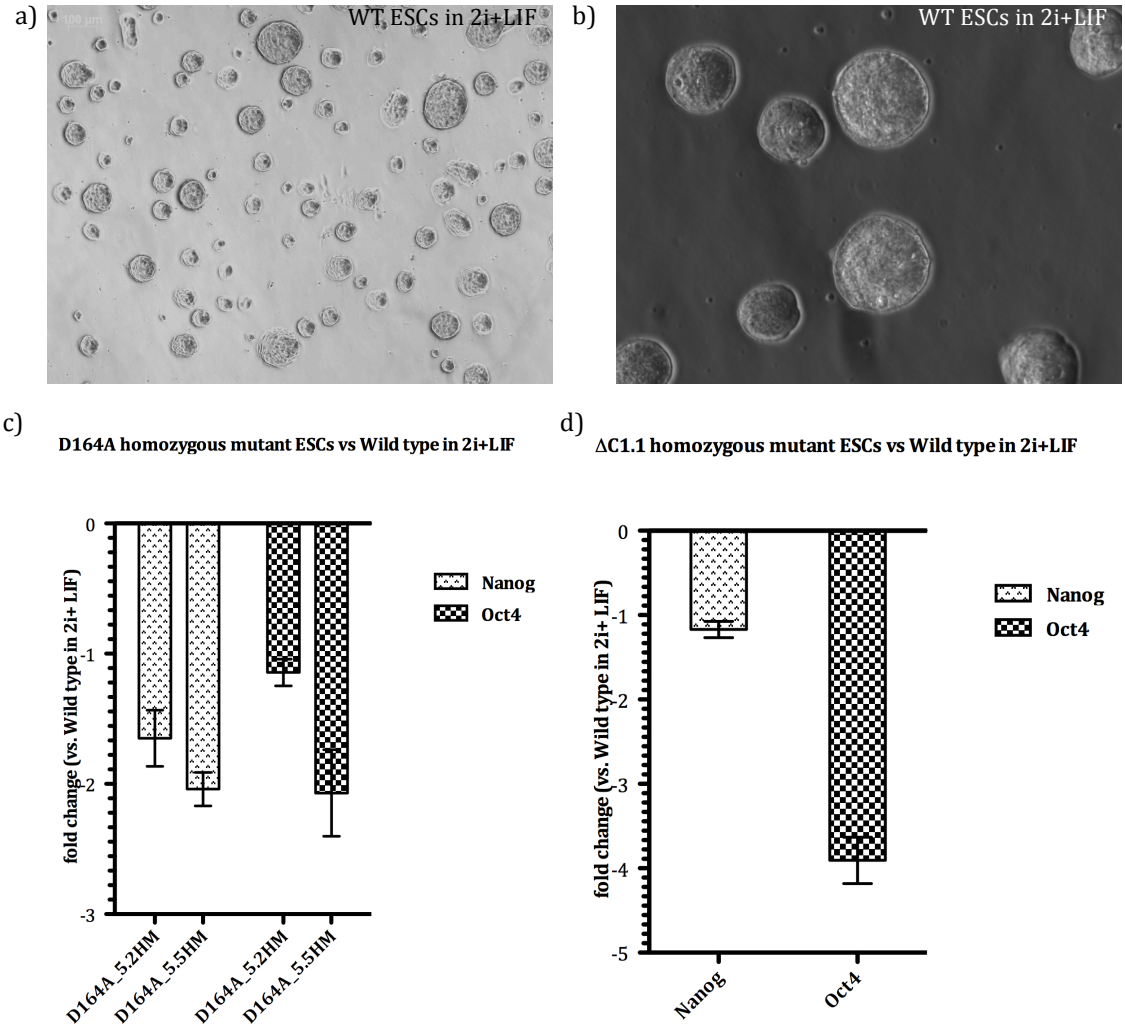

Figure 16: Analysis for pluripotency in N-terminal (D164A) and C-terminal mutant

a) & b) Representation of classical round and compact morphology of WT, N-terminal and C-terminal mutant ESCs in low and high magnification c) & d) Histogram showing expression of pluripotent markers, Nanog and Oct4 in N-terminal and C-terminal β-catenin mutants respectively; down-regulation of Oct4 observed in both the mutants

In vitro differentiation experiments were carried out by embryoid body formation and their analysis using real-time PCR. Embryoid bodies from both N-terminal mutants; D164A_5.2HM and D164A_5.5HM and C-terminal mutant; C1.1 have the classical compact ball-like structure and do not show any cell adhesion defects (Fig. 17a). Interestingly, expression of differentiation markers, *FGF5*, *Gata4* and *T-brachyury* between the two mutants is contrary to each other. Embryoid bodies from the N-

terminal mutants show downregulation of differentiation markers where as the C-terminal mutants show an upregulation (Fig. 17b & 17c). The real-time data for the two N-terminal mutants, D164A_5.2HM and D164A_5.5HM is not consistent and they show variations in the expression of differentiation markers among themselves.

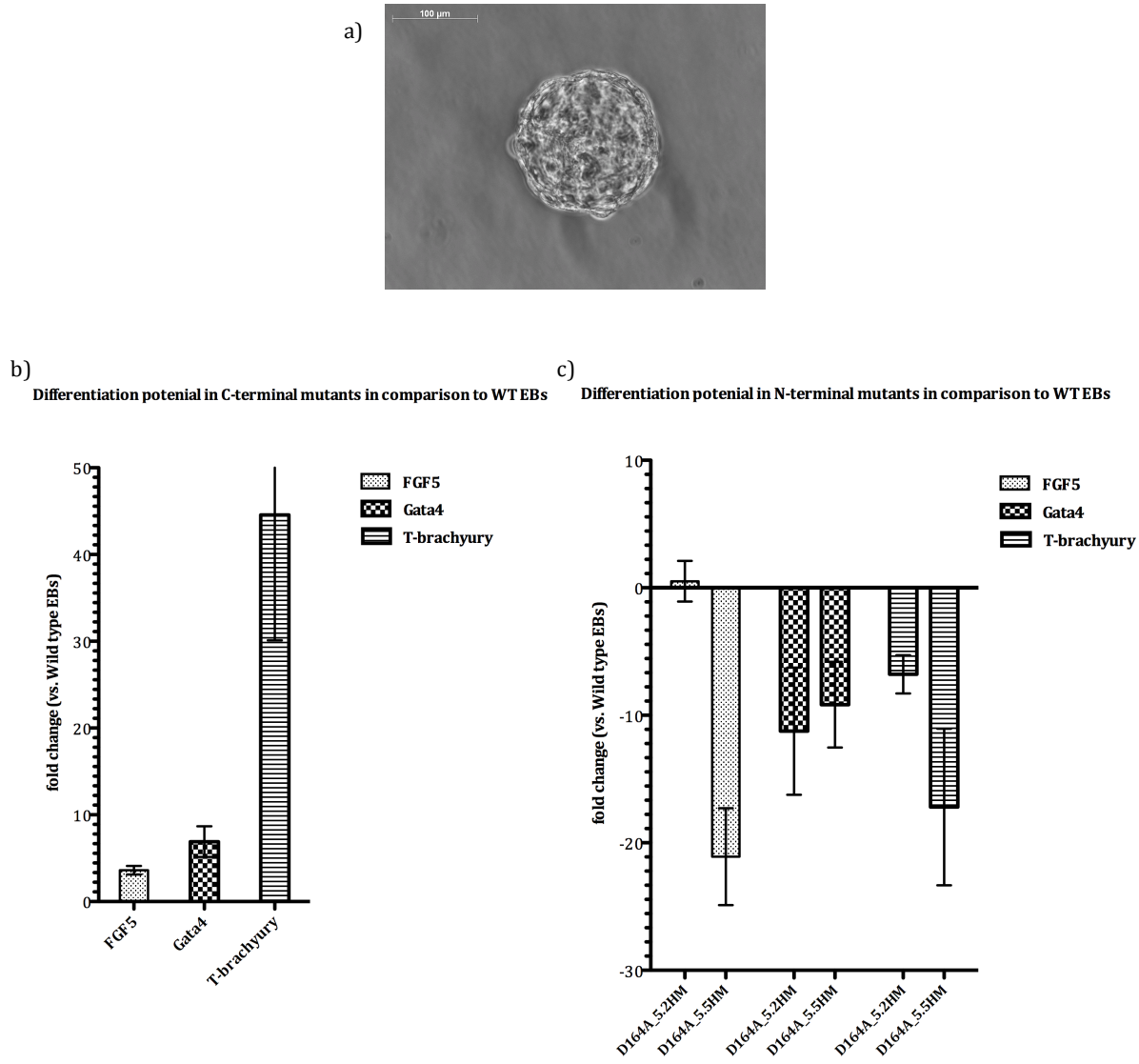

Figure 17: In vitro differentiation of C-terminal and N-terminal β-catenin mutant

a) Representation of a classical compact ball-like embryoid body for both N- and C- terminal mutant ESCs b) C-terminal mutant shows an upregulation of all the differentiation markers specifically T-brachyury which increases by 40 fold c) N-terminal mutant shows downregulation of differentiation markers which are differentially expressed between the two homozygous N-terminal mutants

2.2. *Pramel7* transcription is not directly regulated via β-catenin

Pramel7 has been shown to be a novel downstream target of STAT3 in the LIF/STAT3 pathway. Casanova *et al.* also showed that Pramel7 transcription was impaired when GSK3β was inhibited by CHIR99021 (Casanova et al., 2011). This suggested a role for either GSK3β or its downstream effectors in regulation of Pramel7 independent of STAT3. β-catenin is a known key GSK3β substrate which accumulates in the absence of GSK3β activity. These two findings together suggests a possible regulatory interaction between the key players of Wnt pathway – β-catenin and GSK3β; and Pramel7.

To test this hypothesis, we analysed the expression of Pramel7 at the transcriptional level in β-cat4.2KO ESCs in 2i+LIF conditions, which are the standard culture conditions used in this study. The analysis was performed using real-time PCR. Pramel7 expression was upregulated by 10fold in comparison to WT ESCs. Upregulation of Pramel7 in the absence of β-catenin suggests an inhibitory action of β-catenin on Pramel7. Analysis of Stat3 expression showed no changes in Stat3 at the transcriptional level in the β-catenin4.2KO ESCs (Fig. 18).

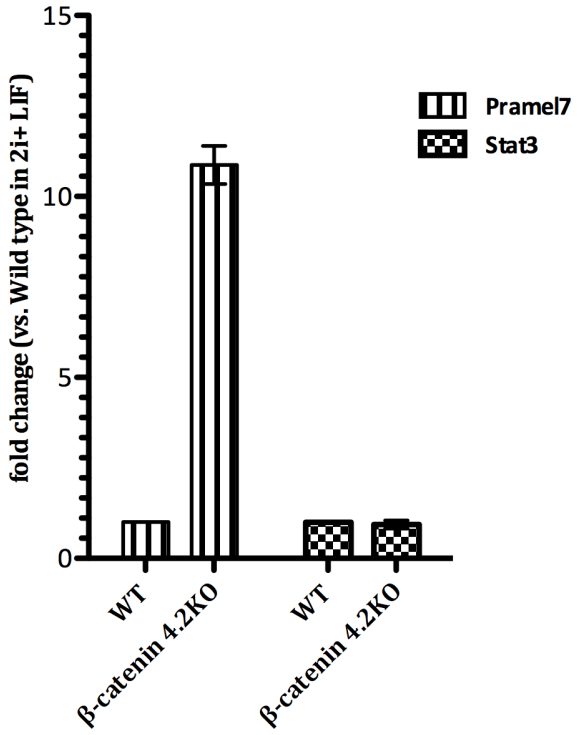

Figure 18: Histogram showing expression of Pramel7 and Stat3 in β-cat4.2KO ESCs

The real time PCR analysis on β-catenin4.2KO ESCs shows an upregulation of Pramel7 by 11 fold in comparison to WT ESCs. No significant changes are observed in the expression of Stat3 in β-catenin4.2KO ESCs when compared to WT ESCs. Levels of mRNA were determined by quantitative real-time PCR and normalized to the housekeeping gene, β-actin. The levels of WT are set as 1.

In order to check if the β-catenin and Pramel7 act antagonistically to each other, we analysed the expression of β-catenin in Pramel7 overexpressing ESCs expanded in 2i+LIF system. No downregulation of β-catenin was observed indicating that Pramel7 did not regulate the transciption of *β-catenin* (Fig. 19).

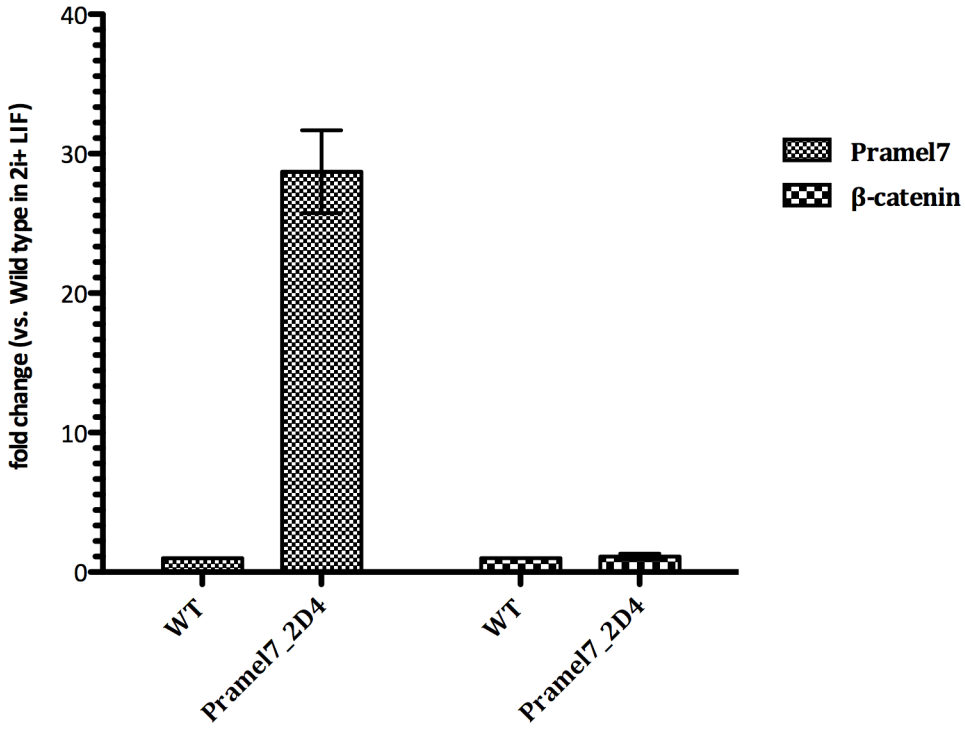

Figure 19: Histogram showing expression of β-catenin in Pramel7 overexpressing ESCs

Pramel7 overexpressing ESCs were labeled Pramel7_2D4. Both WT and Pramel7-2D4 ESCs were expanded in 2i+LIF medium and the expression of β-catenin was checked in comparison to WT ESCs. Overexpression of Pramel7 had no effect on transcription of *β-catenin*. Levels of mRNA were determined by quantitative real-time PCR and normalized to the housekeeping gene, β-actin. The levels of WT are set as 1.

To assess whether overexpression of β-catenin had an effect on transcription of *Pramel7*, we stably transfected wild type ESCs with a "S33Y" vector expressing constitutively active β-catenin harbouring a single point mutation in the phosphorylation site recognised by GSK3β degradation complex (obtained from Prof. Basler group). Successfully transfected clones were named "WT_S33Y". Surprisingly, WT_S33Y clones expressed more Pramel7 in comparison to WT ESCs. Interestingly, evaluating the expression of Pramel7 among the clones showed that Pramel7 reduced upon increase in the transcriptional levels of β-catenin in various clones (Fig. 20). Clones 1 and 2 have 5-10 fold of β-catenin in comparison to wild type and they retain significantly high Pramel7 expression. However, a 15 fold increase in the levels of β-catenin as seen in clone 3 leads to a drastic downfall in the expression of Pramel7.

WT_S33Y ESC clones show an upregulation in Stat3 upon constitutive expression of β-catenin.

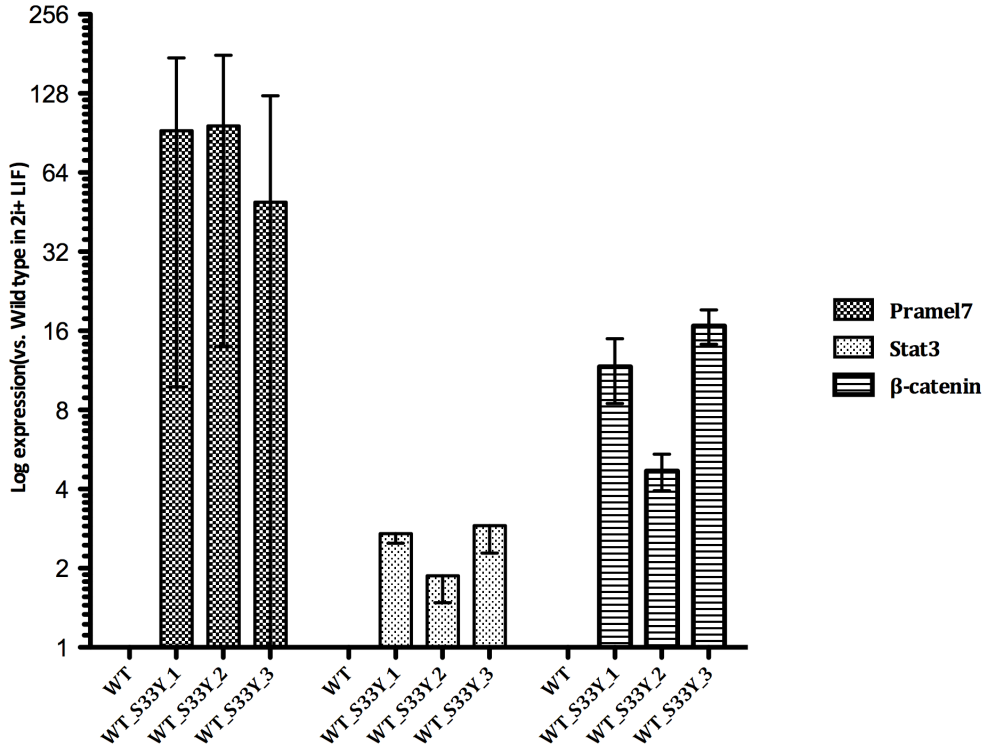

Figure 20: Relative expression of Pramel7 and Stat3 compared to WT ESCs in clones expressing constitutively active β-catenin

The mRNA levels of Pramel7 reduce with the increase in the transcription of β-catenin as observed among three clones showing varied amounts of β-catenin. Levels of mRNA were determined by quantitative real-time PCR and normalized to the housekeeping gene, β-actin. The levels of WT ESCs not transfected with the vector are set as 1.

To determine whether the Pramel7-β-catenin interaction is direct and to map the domain through which it inhibits Pramel7 expression, we generated, as mentioned in the previous chapter, and analysed the expression of Pramel7 in D164A N-terminal mutants and C-terminal mutants. N-terminal mutants showed a variation in the expression of Pramel7 where D164A_5.2HM showed downregulation of it and D164A_5.5HM showed the contrary effect (Fig. 21a). Thus, the result from the two different homozygous mutants was conflicting. Transcription of Stat3 decreased slightly in the N-terminal mutant in comparison to WT ESCs. Intriguingly, C-terminal mutant showed a 4 fold upregulation of Pramel7 even though there was 5 fold reduction in the expression of Stat3 (Fig. 21b). This suggests a role of β-catenin directly or indirectly in the mechanism.

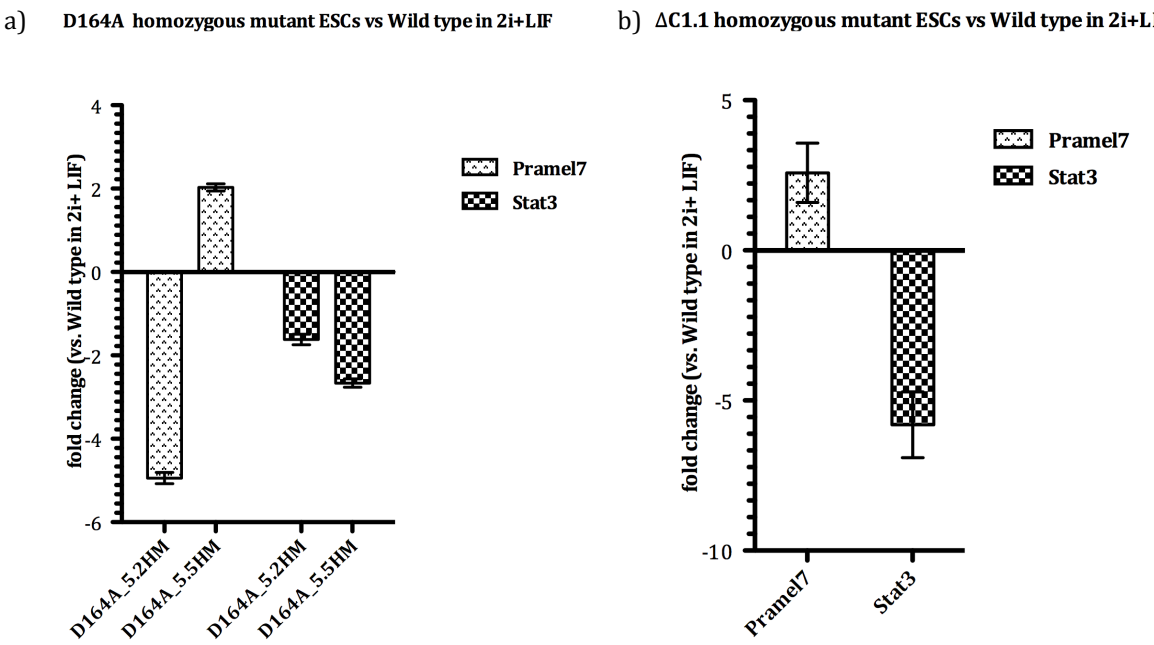

Figure 21: Expression of Pramel7 in N-terminal and C-terminal β-catenin mutants

a) The two N-terminal D164A mutants show variation in expression of Pramel7 b) C-terminal mutation increases Pramel7 transcription by 4 fold where as reduces Stat3 expression by 5 fold. Levels of mRNA were determined by quantitative real-time PCR and normalized to the housekeeping gene, β-actin. The levels of WT ESCs are set as 1.

Taken together, these results indicate that the Pramel7 is indirectly regulated through β-catenin and suggests a role for GSK3β which is a central regulator of β-catenin.

2.3. Effect of GSK3β inhibition on Pramel7 transcription is not mediated via β-catenin

In order to see if it is the absence of β-catenin and/or presence and activity of free GSK3β which is regulating Pramel7 transcription, we monitored Pramel7 expression using QRT-PCR after CH+LIF and LY+LIF induction. CH and LY are small molecule inhibitors. They are selective inhibitors of GSK3β and PI3K respectively. CH inhibits GSK3β by blocking its kinase activity. LY is a potent and cell-permeable inhibitor of PI3K which inhibits ATP binding to the catalytic subunit of PI3K and blocks its kinase activity. This prevents the phosphorylation of GSK3β, thus maintaining its kinase activity.

β-cat4.2KO ESCs were cultured and expanded normally in 2i+LIF. The medium was later changed to N2B27, which is the defined medium without the 2 inhibitors and LIF, for 4 hrs before incubating the cells for 1hr, 5hrs and 24hrs with CH+LIF or LY+LIF.

After 4hrs N2B27, Pramel7 was upregulated in β-cat4.2KO ESCs in comparison to WT ESCs similar to cells cultured in 2i+LIF conditions (Fig. 22a). However, upon stimulation with CH+LIF, Pramel7 level reduced to WT ESCs level (Fig. 22b). In comparison to 4.2KO ESCs in N2B27, CH+LIF stimulated cells again showed downregulation of Pramel7 (Fig. 22c).

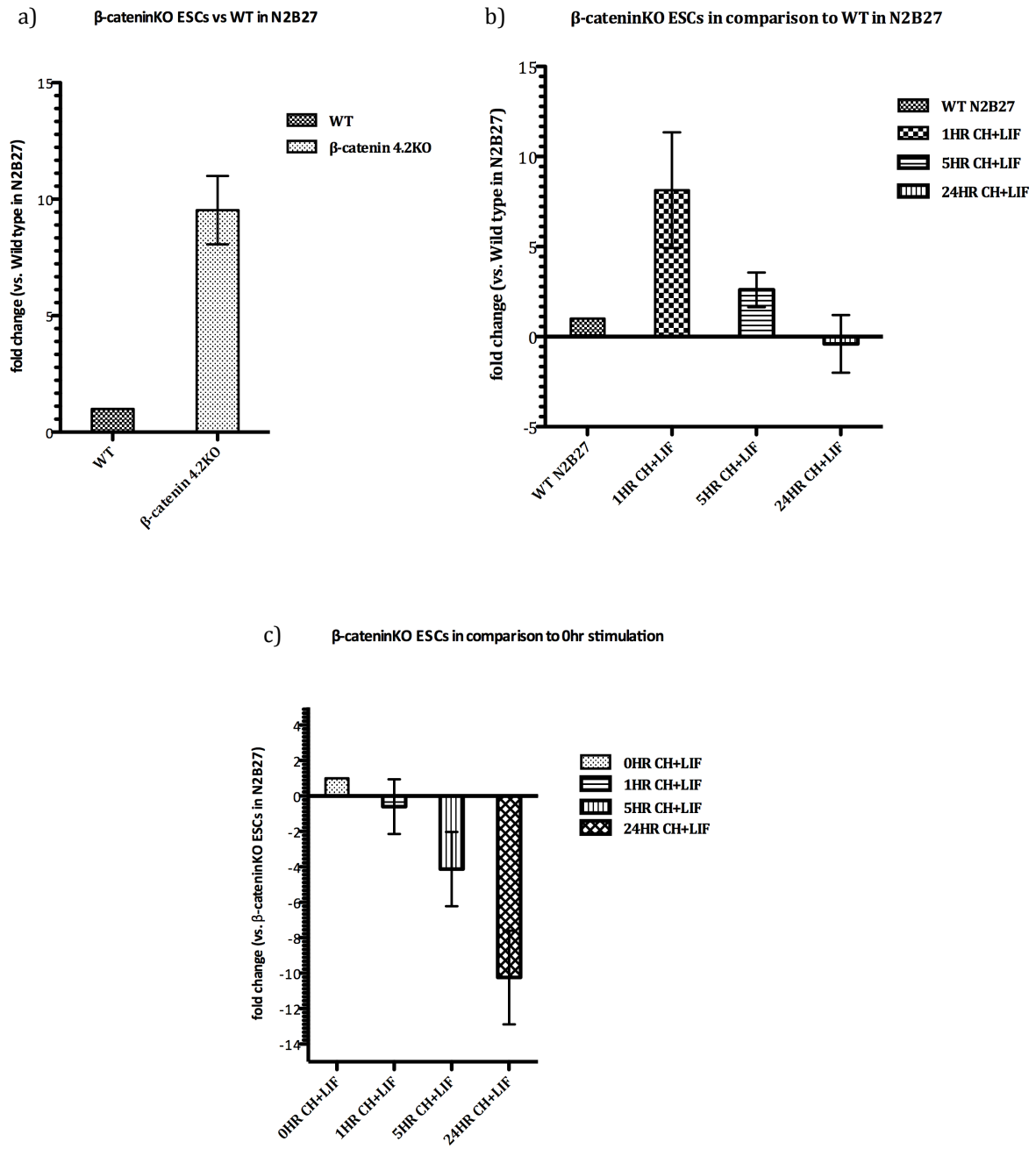

Figure 22: Histogram showing expression of Pramel7 with CH+LIF stimulation in β-cat4.2KO ESCs

a) There is 10fold higher expression of Pramel7 in β-cat4.2KO ESCs in comparison to WT ESCs both cultured in 4hrs N2B27 b) Pramel7 expression in β-cat4.2KO ESCs is similar to WT ESCs after 24hr CH+LIF stimulation Levels of mRNA were determined by quantitative real-time PCR and normalized to the housekeeping gene, β-actin. The levels of WT ESCs cultured in N2B27 are set as 1. c) In comparison to β-cat4.2KO ESCs cultivated for 4hrs N2B27, Pramel7 is 10fold downregulated after 24hrs CH+LIF stimulation. Levels of mRNA were determined by quantitative real-time PCR and normalized to the housekeeping gene, β-actin. The levels of β-cat4.2KO ESCs cultured in N2B27 are set as 1.

Surprisingly, cells stimulated with LY+LIF did not show strong upregulation in expression of Pramel7 compared to 0hr stimulation (Fig. 23).

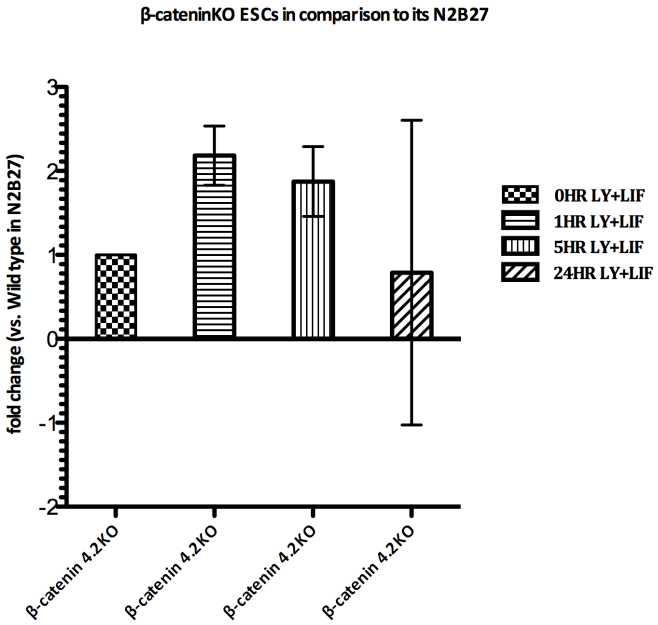

Figure 23: Histogram showing expression of Pramel7 in β-cat4.2KO ESCs upon LY+LIF stimulation

No massive upregulation of Pramel7 was observed in presence of LY+LIF. Histogram shows an initial increase in Pramel7 by 2 times which gradually decreases after 24hrs LY+LIF

However, CH timepoint analysis clearly suggests that inhibiting the activity of GSK3β affects the transcription of Pramel7 in β-cat4.2KO ESCs.

To assess if the changes in expression of Pramel7 in β-cat4.2KO ESCs after CH+LIF stimulation is due to differential phosphorylation of STAT3 in these ESCs, we analysed the β-cat4.2KO ESCs for phosphorylation of STAT3 at Y705 by performing Western blot with antibodies for Y705 phosphorylation. Phosphorylation of STAT3 at Y705 residue plays an important role for dimerisation, nuclear translocation and activity of STAT3 to regulate the expression of its downstream target genes like *Pramel7*. There were no changes in the phosphorylation of STAT3 compared to WT ESCs except the 24hr timepoint. In the 24hr timepoint, a weak band for phosphorylation of STAT3 is still observed in WT ESCs unlike the β-cat4.2KO ESCs which show absence of it after 24hrs (Fig. 24). Overall, this experiment suggests that the regulation of Pramel7 in β-cat4.2KO ESCs in presence of CH+LIF is independent of STAT3 phosphorylation.

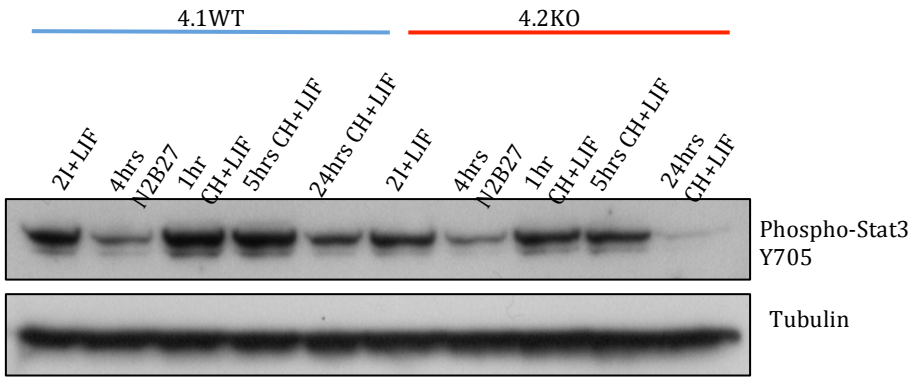

Figure 24: Immunoblot for β-cat4.2KO ESCs using antibodies against Stat3 Y705 phosphorylation and Tubulin

To check if there is a link between β-catenin, GSK3β and Pramel7, we stimulated the WT ESCs as per above protocol with N2B27+ CH only. It was observed that Pramel7 expression was 2 fold higher after 1hr CH stimulation but was gradually downregulated with longer time points (Fig. 25).

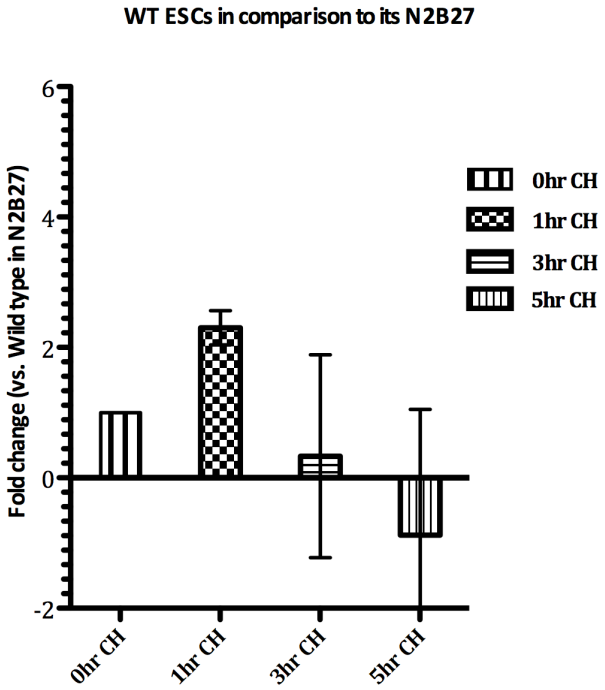

Figure 25: Pramel7 expression in WT ESCs upon CH stimulation

This gives a clear indication that upregulation of Pramel7 in β-catKO ESCs is due to the activity of free GSK3β, not bound to β-catenin for degradation. However, a clear link between the trio is still not confirmed.

2.4. Transcription of *Pramel7* is dependent on the presence of GSK3β

To confirm the hypothesis that it is the presence of GSK3β that regulates Pramel7, we performed a knockdown experiment in WT ESCs cultured in 2i+LIF conditions. We transfected WT ESCs with siRNAs against GSK3β and confirmed the specific knockdown by QRT-PCR analysis. We, then investigated the expression of pluripotency markers and Pramel7 at the transcriptional level. Transcription of Pramel7 was completely abrogated indicating the importance of the presence of GSK3β protein in its regulation. However, the ESCs still expressed markers of pluripotency, Nanog and Rex1 (Fig. 26).

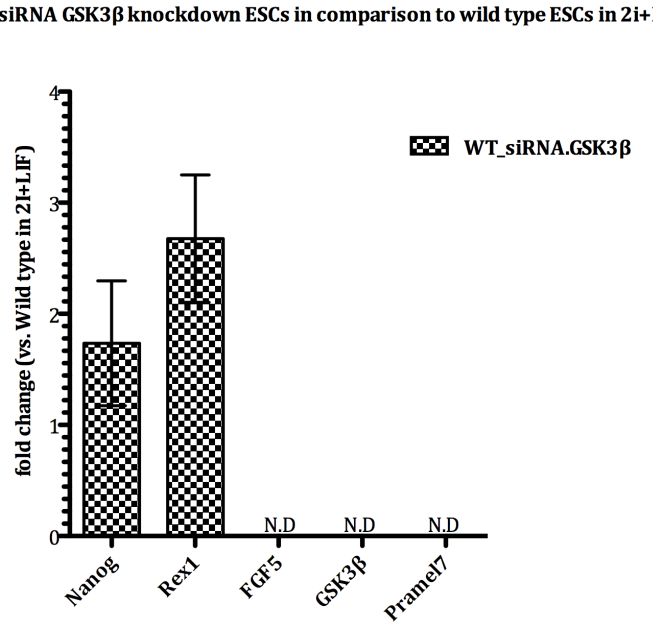

Figure 26: Expression analysis for siRNA knockdown of GSK3β

Absence of GSK3β expression upon siRNA transfection confirms the knockdown of GSK3β. WT_siRNA_GSK3β ESCs express the pluripotency markers, Nanog and Rex1 and lack FGF5 expression, a differentiation marker. In comparison to WT ESCs, transcription of Pramel7 is completely blocked in the absence of GSK3β. Levels of mRNA were determined by quantitative real-time PCR and normalized to the housekeeping gene, β-actin. The levels of WT ESCs cultured in 2I+LIF are set as 1.

To further assess the dependence of Pramel7 expression on the presence and activity of GSK3β, we examined induction of Pramel7 in WT ESCs on addition of either CH99021, PD0325901 (PD), or both after incubating the cells for 4hrs in N2B27 only. PD is a potent and selective inhibitor of Erk kinase. We confirmed the observation that Pramel7 regulation is blocked in presence of CH. We observed that Pramel7 was upregulated in the presence of PD and GSK3β suggesting that it is the activity of GSK3β and inhibition of Erk modulating the expression of Pramel7 (Fig. 27).

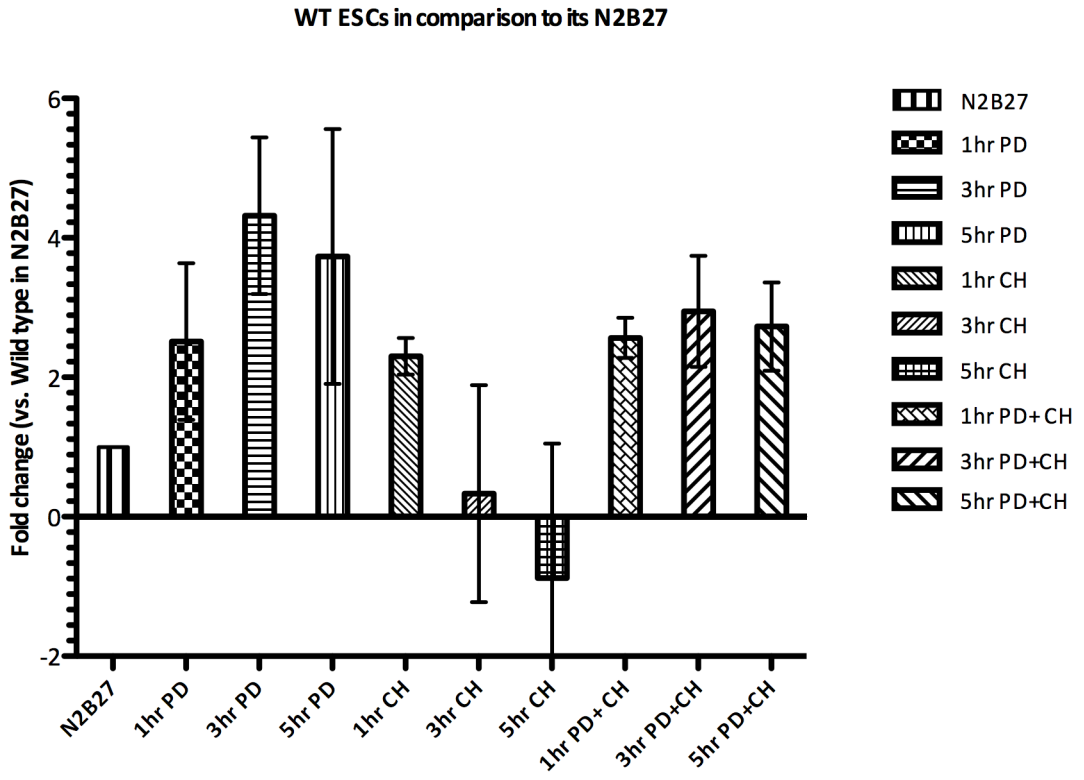

Figure 27: Pramel7 regulation in WT ESCs upon stimulation with PD, CH and PD+CH

There is an upregulation of Pramel7 by 4 fold when stimulated with PD in the presence of GSK3β and reduced expression with CH stimulation in WT ESCs. The levels of WT ESCs cultured in 2I+LIF are set as 1.

To test the robustness of this hypothesis, we then carried out a timepoint experiment on the following GSK3β mutants described in Materials and Methods 2.1.3 section. The mutants DKO, DKO_GSK3β and DKO_K85A ESCs obtained from Prof. Doble (Kelly et al., 2011) which were generated and expanded in serum+LIF conditions. The above three cell lines were incubated for 24hrs in standard ESC medium containing serum and without LIF. They were then stimulated with PD only to simulate the effect of absence, presence of GSK3β and activity of GSK3β. Pramel7 was downregulated in the presence of PD in DKO ESCs (Fig. 28b). It was maintained at the wild type levels or showed slight upregulation in DKO_GSK3β and DKO_K85A in the presence of PD (Fig. 28c & 28d). Intriguingly, in comparison to DKO ESCs, expression of Pramel7 was reduced in DKO_GSK3β and upregulated in kinase inactive, DKO_K85A (Fig. 28a). All the mutants were cultured in normal ESCs medium with serum and LIF. This shows an inclination towards the presence of GSK3β rather than its activity in ESCs cultured in serum+LIF conditions.

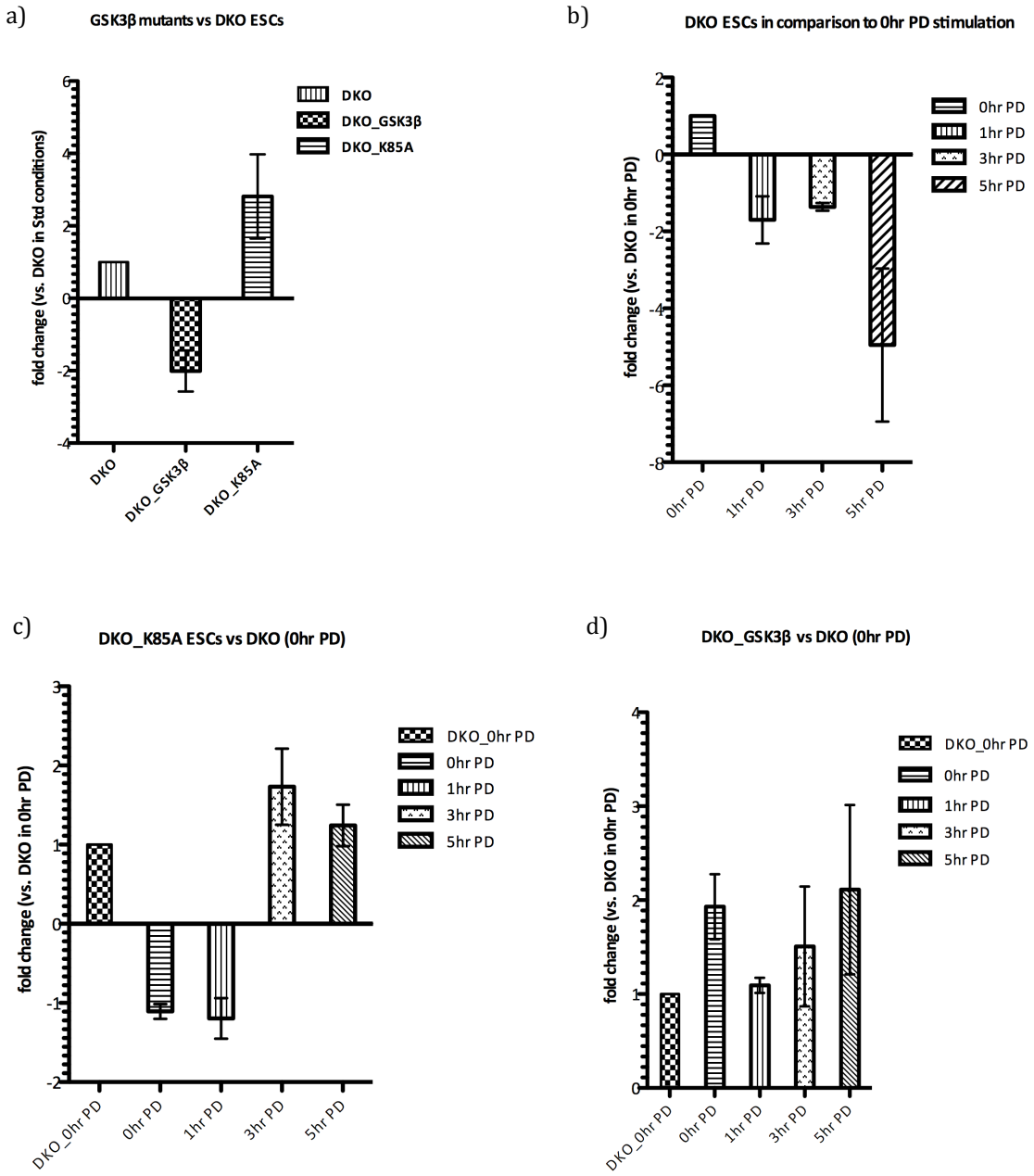

Figure 28: Expression of Pramel7 upon PD stimulation in different GSK3β mutants

a) In comparison to DKO ESCs, kinase inactive DKO_K85A ESCs show slight upregulation of Pramel7 unlike DKO_GSK3β b) DKO ESCs show 5 fold downregulation of Pramel7 when stimulated with PD c) & d) Both the GSK3β mutants retain the expression of Pramel7 in presence of PD

Surprisingly, real-time analysis performed on DKO ESCs showed an upregulation of Pramel7 in comparison to E14 ESCs in standard ESC medium (Fig. 29). However, this can be a compensatory mechanism seen in DKO ESCs discussed later.

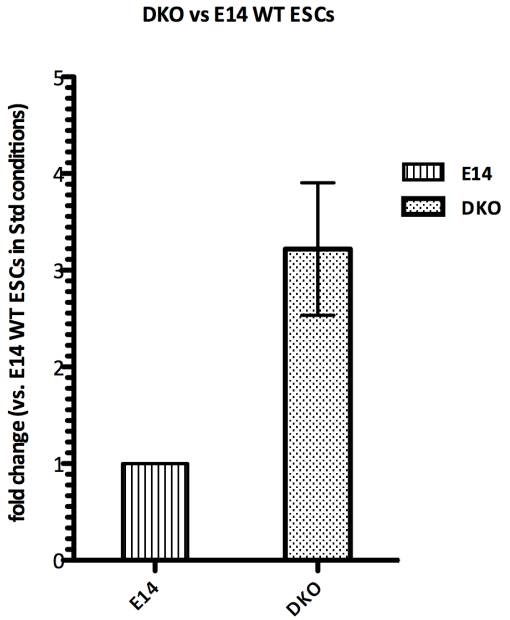

Figure 29: Upregulation of Pramel7 in DKO ESCs compared to E14 WT ESCs

Altogether, these results indicate that GSK3β is essential for transcriptional regulation of Pramel7.

2.5. Regulation of Pramel7 does not depend on physical interaction between STAT3 and GSK3β

In order to address whether Pramel7 physically interacts with GSK3β, we transfected HEK cells with Flag-tagged Pramel7 vector and performed a pull down experiment using antibodies against Flag epitope(performed by Urs Graf). The precipitated material was purified and the eluate was analysed by Western blot with antibodies against Flag, total GSK3 and total STAT3. Neither GSK3β nor STAT3 was co-precipitated with Flag-tagged Pramel7 (Fig. 30). The result suggested that there was no physical interaction between Pramel7 and GSK3β. No physical complex was formed between Pramel7, GSK3 and STAT3.

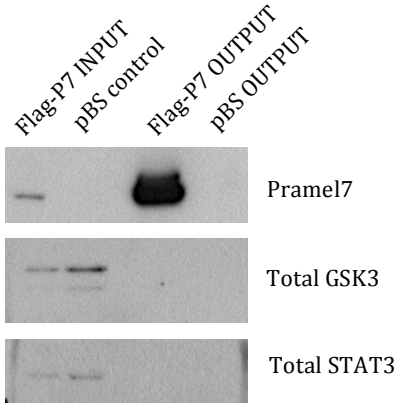

Figure 30: Immunoblot analysis for Flag-IP done with HEK cells for Pramel7

HEK cells were transfected with Flag- tagged Pramel7 vector and pBS vector was used as control. Flag-P7 input shows that the transfection was successful. The pull down eluate analysed by Western blot neither shows a physical interaction between Pramel7 and GSK3 nor Pramel7 and STAT3.

From previous findings from our lab, it is known that Pramel7 is a downstream target of Stat3 and the above results suggest a role of GSK3β in regulation of Pramel7. To gain insight into the mechanism behind regulation of Pramel7, we next investigated possible interactions between STAT3 and GSK3β. We carried out co-immunoprecipitations experiments both in HEK cells and ESCs. The HEK cells were transfected with expression vector for HA-tagged GSK3β_S9A which has constitutively active GSK3β. Immunoprecipitation was performed with an antibody directed against HA-epitope of GSK3β and precipitated material was subjected to Western blot anaysis with anti-GSK3 and anti-STAT3 antibodies. As shown in the Fig. 24, STAT3 did not co-precipitate with GSK3 indicating that they do not form a physical complex. The co-immunoprecipitation protocol was then repeated in DKO used as negative control and DKO_GSK3β ESCs where GSK3β is Flag-tagged. The pull down experiment was performed using antibodies

against Flag-epitope of GSK3β and output was analysed with antibodies against GSK3β and Stat3. It was confirmed that there was no physical interaction between the two proteins (Fig. 31).

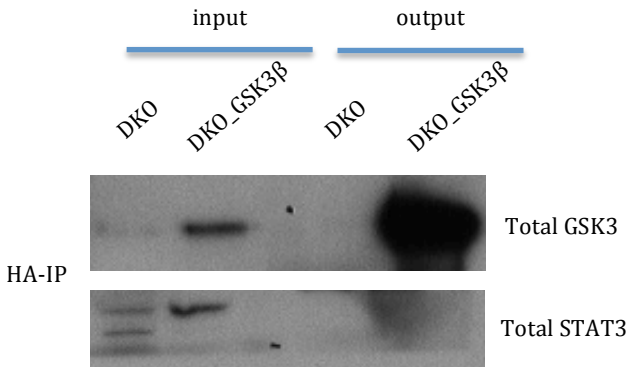

Figure 31: Immunoblot analysis for HA-IP done on HEK cells expressing GSK3β_S9A and DKO_GSK3β ESCs using antibodies against Total GSK3 and Total Stat3

Phosphorylation and dimerization of STAT3 is important for the transcriptional activity of STAT3 on its target genes. To assess whether absence of GSK3β influences phosphorylation of STAT3 both on Tyr705 and Ser727, we investigated the phosphorylation trend in DKO and E14 wild type ESCs upon LIF stimulation. The cells were cultured in standard ESCs medium containing serum without LIF for 24hrs and then subjected to 1hr, 3hrs and 5hrs LIF incubation. Analysis was performed by Western blot using antibodies against specific STAT3 phosphorylations. No changes were observed in the STAT3 phosphorylation between the two cell types suggesting that Pramel7 regulation via GSK3β is independent of STAT3. Thus, the two proteins regulate Pramel7 transcription independent of each other.

C. Discussion

1. Project I: Establishment of pluripotent germline competent rat embryonic stem cells from Brown Norway strain

Rat is the most extensively used laboratory animal in biomedical research particularly useful as a surrogate for studying human diseases. Establishment of first authentic rat ESCs is a remarkable achievement that makes it possible to develop genetic models in the rat, using gene targeting and other technologies that are readily available for mouse ESCs. However, to be successful at this objective, there are a number of challenges that must be overcome. Most importantly, rat ESC culture system is in its infancy and a huge amount of work still needs to be done to be able to realize the full potential of rat ESCs in genetic research. It is crucial to know that before the optimization and standardization of culture conditions for mESCs, it was believed that only early passage cells were germline competent and useful for gene targeting (Brook and Gardner, 1997). The same applies for the rat ESCs and it is essential to deeper understand the underlying stem cell biology of rat in order to sustain pluripotency and produce germline-competent rat ESCs. Brown Norway (BN) rat is a widely known strain and an indispensible tool in experimental medicine and drug development and used extensively for other scientific research purposes. The BN rat sequence was the third mammalian genome to be completely deciphered which makes it a useful candidate for comparing with human and mouse genomes (Gibbs et al., 2004). Generation of pluripotent ESCs from BN strain will be particularly useful for rat functional genomic studies and genetic disease remodeling. The knowledge of genome sequence makes it a better candidate for gene targeting as well. Taking this into consideration, BN rat was chosen as the model for the establishment of pluripotent germline-competent ESCs.

1.1. Derivation and establishment of rat ESCs

Buehr *et al* (2008) had successfully established germline-competent ESCs from Dark Agouti (DA) strain using 3i and 2i medium both in the presence of LIF producing DIA-M feeders and feeder free respectively (Buehr et al., 2008). Derivation of BN ESCs for the study was successfully performed by introducing a few modifications in the protocol published by Buehr *et al* (2008). In our initial derivation experiments from BN rats, there were concerns regarding poor adherence of single cells to the feeders upon

dissociation of the initial outgrowths from the inner cell mass and thus inability to develop ESC colonies. This could have been either due to the strain of feeders used, the substrate used to coat the plates or the method of dissociation of the outgrowths. It is known that addition of LIF enhances the derivation of mouse ESCs (Ying et al., 2008). Normally, mitotically inactivated mouse fibroblasts are used as feeders to support the growth of the ESCs. However, as a source of LIF we used, SNL feeders derived from STO cell line expressing LIF at an abundant level. The feeders also supported the attachment of the inner cell mass for outgrowth development. Replacing gelatin with laminin to coat the plates and using Accutase instead of trypsin for the gentle enzymatic dissociation of the colonies also promoted the attachment of the ESCs. However, laminin was only used in the initial steps of derivation and expansion of early passages to enhance the development of colonies. The above modifications supported the attachment and proliferation of the dissociated single cells as tightly packed round compact colonies. Thus, the abundance of LIF and the use of laminin and Accutase led to the successful establishment of five ESC lines in 2i medium and the efficiency was improved from 15% to 55% (Table 3). I also tried to establish rat ESCs from a single round of breeding of Lewis rat strain using the optimized protocol above. Interestingly, the ICMs plated on feeders could form outgrowths but failed to adhere to the feeders and develop colonies upon dissociation. Studies on mESCs have shown discrepancies in derivation efficiency of ESCs from different strains. Unlike 129 strain, mESCs from non-permissive and recalcitrant strains like CBA and C57/B6 can be successfully derived only by inhibition of MEK/ERK in addition to the LIF suggesting an intrinsic difference within the epiblast to sustain pluripotency (Batlle-Morera et al., 2008). This indicates that the efficiency of rat ESC derivation may be strain-dependent similar to mESCs. Investigations into the differences in various signaling pathways, mentioned in the introduction, between the two species will be essential to create stabilized and optimized ESC establishment protocol for rats.

1.2. *In vitro* and *in vivo* characterization of rat ESCs

Expression of key pluripotency genes such as *Oct4* and early embryonic stage markers, SSEA-1 and alkaline phosphatase is an essential preliminary screening tool for pluripotency. All the ESC lines were positive for the above markers (Fig. 9). Investigation of the differentiation potential of the BN ESC lines confirmed their ability to differentiate *in vitro* upon response to differentiation stimuli and produce teratomas *in vivo* (Fig. 10 &

Fig. 11). However, upon differentiation, high proportion of cell death was observed in BN_6.1 and BN_6.2 ESC lines with quite a few cells attached. This made the analysis with immunofluorescence staining of differentiation markers challenging in these lines. These differences between the lines may be associated with the inherent genetic variation of the embryos from which the ESCs were derived.

1.3. Generation of germline-competent chimeras

The utmost proof of authenticity of ESCs is their capacity to colonise host embryos and contribute to differentiated progeny of the three germ layers and hence produce germline-competent chimeras. Lewis rat blastocysts were used as the recipient embryos for injecting BN ESCs and Wistar females were taken as the foster ones. Only 1 live pup showing no chimerism was obtained from the blastocyst injection (Table 5). Generation of just one pup from 108 injections performed points towards low viability of the embryos rather than the authenticity of ESCs. Thus, the handling issues related to blastocyst injection and embryo transfer technique might have been the potential cause for the failure to generate chimeras.

A very narrow time window exists in embryonic development for the ES cell injection procedure to be successful in the generation of chimeric mice. Therefore, harvesting, injection, and surgical transfer of the rat blastocysts are all performed the same day and these are the three main areas where problems can be encountered. Harvesting the embryos and surgically reimplanting them depend on the rat strain, the laboratory conditions, and the conditions in the animal facility. The latter two are quite complex to resolve. As mentioned earlier, Lewis rat were used as the donor strain for blastocyst injections. Regarding the embryo collection, the Lewis strain is reported to have small litters. Thus, only a small number of blastocyst stage embryos are obtained for injections. Unlike the mice, the superovulation protocols applied do not work very well with the rats. Hence, it was not possible to superovulate the rats to increase the number of embryos for blastocyst injection. Blastocyst injections should also be performed very meticulously in order to prevent any damage to the embryos. It has to be done fast enough to transfer the embryos back into the 37°C incubator to improve the chances of obtaining viable and healthy embryos to transfer. Regarding the ESCs, use of an appropriate culture medium along with incubation at the right temperature are factors required to optimize the survival of ESCs. Thus, it is possible that retaining the ESCs and embryos at RT for injections for longer periods of time reduced the viability of the

injected ones. With respect to the technique, there are other factors that have been shown to affect the efficiency of chimera generation in mice and might also hold true for rat ESCs. One of these factors is the activation of ESCs, to synchronise the ESCs in the log phase of growth, by changing the ESCs culture media just 2 hrs before the injection. This has been shown to increase the efficiency of chimera generation by giving the ESCs a competitive advantage over the endogenous blastomeres of the ICM (Ramírez et al., 2009). Another study by Huang *et al* (2008), suggested that the culture media might produce damage in the injected ESCs that could result in a leak of their cytoplasm into the host embryo thereby poisoning it and affecting the viability of the embryos (Huang et al., 2008). To summarise, blastocyst injection and embryo transfer technology are difficult and time-consuming techniques that require a huge amount of practice and are most efficient when performed by skilled injectionist.

In general, several factors such as the genetic background of the ESCs, stemness, karyotype, pathogen status of the ESC line and genetic background of the recipient embryo can have an affect on the ability of the ESCs to colonize host embryo to generate chimeras and to exhibit competence in transmitting their genetic material through the germline. Various groups have established ESCs from different rat strains namely Brown Norway (BN), Wistar (WI), Dark Agouti (DA), Sprague-Dawley (SD) and Fischer 344 (F344) strains (Zhao et al., 2010) (Hirabayashi et al., 2010) (Li et al., 2008) (Buehr et al., 2008). However, DA ESCs are the only germline-competent ESCs generated to date. In parallel to our study, Zhao *et al* had published a study showing derivation of ESCs from BN rats in 2010, where they injected the SD blastocysts with BN ESCs resulting in generation of chimeric animals which failed to produce offsprings with the BN genetic background (Zhao et al., 2010). Hence, the genetic background of the ESCs used in this study can be another factor to consider. Confirming expression of pluripotency genes as well as maintainance of normal karyotype in ESCs are essential factors to consider in order to eradicate the cell lines that may negatively affect their germline transmissibility. Chromosomal anomaly is one of the major causes for failure of germline competence in mESCs (Liu et al., 1997) (Suzuki et al., 1997). The established rat ESC lines show 70% normal karyotype and it is possible that a higher rate may be necessary in the BN strain to generate chimeras and demonstrate germline competency in rats. It is also possible that the injected ESCs might have problems in proliferating and developing into various organs during embryogenesis due to the undergone enzymatic treatment before injections. It has been shown that the presence of virus, bacteria or *Mycoplasma*

affects germline transmission in mESCs (Mahabir et al., 2009) (Markoullis et al., 2009). However, this was not the reason in our case. Lastly and most importantly, the genetic background of recipient embryos hugely affects the generation of chimeras and germline transmission. Relatively little is known about the compatibility between the rat ESCs and the host blastocyst genetic background. Ideally, the host genetic background should provide ESCs with an optimal developmental advantage when injected into the blastocysts. This allows the ESCs to contribute to germline-competent chimeras (Li et al., 2008) (Zhao et al., 2010) (Tong et al., 2011). Several groups have generated rat ESC germline-competent chimeras in the combinations DA: F344 (Li et al., 2008), WI: WI and DA-WI (Hirabayashi et al., 2010), WI: LEA, WI: WI, LEA: WI, WI-LEA: WI (Kawamata and Ochiya, 2010), SD: DA-SD (Men et al., 2012) and DA: SD (Hong et al., 2012). Except the Li *et al.* and Hong *et al*., all the other studies derived rat ESCs from transgenic rats. In a personal communication with Aron Guerts group, it was mentioned that BN ESCs were very hard to contribute to germline competent chimeras due to the lack of compatible hosts to the BN ESCs. No study has been reported to date where Lewis blastocysts are used as the host embryos and we were the first ones to inject BN ESCs into the Lewis donor blastocysts. As mentioned earlier, the compatibility of the ESCs and the host embryo plays a huge part in the generation of chimeras showing germline transmission. Hence, it can be suggested that the host-ESCs combination used was not ideal. Thus, our BN ESCs fall into the category of established rat ESCs that have not shown germline competency to date.

2. Project II: Elucidating the molecular mechanisms regulating transcription of Pramel7 in mouse embryonic stem cells

2.1. Link between Pramel7 and β-catenin:

In accordance with the recent publication from Lyashenko and colleagues, we show by generation of β-catenin ESCs that β-catenin is dispensable for self-renewal of ESCs and show no alteration of self-renewal markers (Lyashenko et al., 2011). However, contradictory to Lyashenko *et al* (2011) and Wray *et al*, (2011) our data does not demonstrate that β-catenin ESCs are LIF-dependent (Lyashenko et al., 2011) (Wray et al., 2011). This can be due to the different protocols used to generate and propagate the two β-catenin deficient ESC lines. The β-catenin null ESC lines were generated by Cre-mediated recombination and cultured in serum conditions in Lyashenko and colleagues study unlike the ones generated in our studies, which were generated by breeding heterozygous β-catenin mice and cultured in 2i+LIF conditions. A study by Hao *et al* showed that expression of Stat3 was activated by β-catenin (Hao et al., 2006). Real-time analysis showed no differences in Stat3 expression between the β-catenin KO ESCs and wild type (Fig. 18), thus it is not required for Stat3 transcription. However, an upregulation of Stat3 was observed in constitutively active β-catenin S33Y ESCs in accordance with Hao *et al* (Hao et al., 2006).

In the result section, we observe that Pramel7 is upregulated in β-catenin KO ESCs in comparison to the WT ESCs in 2i+LIF conditions (Fig. 18) and overexpression of Pramel7 has no effect on transcription of β-catenin (Fig. 19). Thus, the two do not act antagonistically to each other and β-catenin seems to regulate transcription of Pramel7 directly or indirectly via GSK3β as inhibition of GSK3β in WT ESCs hinders Pramel7 regulation.

Regulation of GSK3 occurs via multiple signalling pathways. It functions by phosphorylating its targets resulting in their inactivation (Doble and Woodgett, 2003). In a resting cell, it exists in a constitutively active form and is negatively regulated in response to a cell signal. Its kinase activity is inhibited by phosphorylation of its N-terminal serine residue, ser9 (McManus et al., 2005). One of the kinases involved in the phosphorylation of GSK3β is Akt/PKB (Doble and Woodgett, 2003). Another model in which the inhibiting role of GSK3β is blocked is the Wnt signalling pathway. When exposed to the Wnt ligands, β-catenin escapes the phosphorylation by GSK3β and translocates to the nucleus. Transmission of Wnt signal after binding of Wnt ligand to

the Frizzled receptor and LRP5/6 requires the GSK3 mediated phosphorylation of LRP5/6. The mechanism behind this process is poorly understood. However, binding of GSK3β along with the rest of the degradation complex to the membrane prevents degradation of β-catenin. However, the two mechanisms regulating GSK3β work independent of each other. PKB mediated inhibition of GSK3β does not lead to stabilization of β-catenin and active Wnt signalling does not result in phosphorylation of GSK3β on Ser9 thus does not decrease the phosphorylation of its other targets (McManus et al., 2005) (Doble et al., 2007). Thus, there might be two different pools of GSK3β for the above signalling pathways. CH is a specific inhibitor of GSK3β, which mimics the effect of active Wnt canonical signalling. But, the mechanism behind the inhibition of GSK3β by CH is not well documented. It has been shown in WT ESCs that CH reduces β-catenin phosphorylation thus promoting the activity of β-catenin (Ying *et al* 2008). We observed an upregulation of Pramel7 in β-catenin KO ESCs in comparison to WT ESCs in 2i+LIF conditions and downregulation of it when the clones were compared. Thus, expression of Pramel7 was reduced in a dose dependent manner in the presence of constitutively active β-catenin (Fig. 20). It can be said that presence of β-catenin has an inhibitory effect on Pramel7 transcription. Further experiments carried out on the N-terminal mutants showed discrepancies in the results and made the data difficult to interpret (Fig. 21a). C-terminal mutant shows an upregulation in Pramel7 expression (Fig. 21b). This suggests a potential role of the C-terminal region of β-catenin in the regulation of Pramel7. Nevertheless, this observation is based on a single ESC line carrying the C-terminal mutation, which hinders the judgement of the results. Generation and analysis of higher number of mutant ESCs might help to overcome this issue. However, it was very laborious to derive ESCs from the β-catenin mutant embryos, specifically the ones carrying the C-terminal mutation, as the heterozygous pregnant females usually have a uteri- vaginal defect. Activity of the C-terminus is essential for mesoderm formation (Valenta et al., 2011) and this can be a likely reason for the defective development of uterus in the heterozygous mice. However, the results obtained from β-catenin KO ESCs pinpoints that, in the absence of β-catenin, the pool of free GSK3β might be involved in the transcriptional regulation of Pramel7.

2.2. Link between GSK3β and Pramel7

In order to see if it is the absence of β-catenin and/or presence and activity of free GSK3β which is regulating Pramel7 transcription, we monitored Pramel7 expression using QRT-PCR after CH+LIF and LY+LIF induction. CH and LY are small molecule inhibitors of GSK3β and PI3K respectively. In CH+LIF conditions, independently of presence or absence of β-catenin, we observe a downregulation of Pramel7 transcription (Fig. 22 & Fig. 25). However, stimulation of β-catenin KO ESCs with LY+LIF showed no changes in transcription of Pramel7 (Fig. 23). Reduction of Pramel7 after 24 hrs might be due to LY toxicity (Fig. 23). These findings pinpoint GSK3β as a central player in the mechanism regulating Pramel7 and poses new questions. Inhibition of GSK3β by CH either upregulates one of the GSK3β targets, which can be a potential transcriptional repressor of Pramel7 or it downregulates Stat3, the transcriptional activator of Pramel7. However, western blot analysis on β-cateninKO ESCs stimulated with CH+LIF showed no changes in phosphorylation of Stat3 at tyrosine residue in comparison to WT ESCs (Fig. 24). Thus, Pramel7 regulation via GSK3β is independent of LIF/STAT3 pathway.

Knockdown of GSK3β using siRNA resulted in complete abrogation of transcription of Pramel7 (Fig. 25) in contrast to its downregulation when the activity of GSK3β was inhibited by CH (Fig. 22 & Fig. 25). Ying *et al* (2008) confirmed the inhibition of GSK3β by CH by interrogating DKO ESCs lacking both GSK3α and GSK3β. It has been shown that DKO ESCs show a constitutive TOPFLASH (Wnt/ β-catenin transcriptional reporter) activation 50 fold higher than CH treated wild type ESCs (Ying et al., 2008). Thus, it is known that CH does not completely inhibit GSK3β and therefore, the ESCs in our study still express Pramel7 in presence of CH. Expression of Pramel7 was analysed in DKO ESCs to replicate the result obtained with GSK3β siRNA knockdown. Intriguingly, DKO ESCs in comparison to WT ESCs showed an upregulation of Pramel7 in serum+LIF conditions (Fig. 29). However, this can be attributed to the compensatory mechanisms of the cell to maintain itself pluripotent, most likely through regulation of Pramel7 by LIF/STAT3 pathway.

WT and various GSK3β mutant ESCs were then stimulated with PD only to simulate the effect of absence, presence of GSK3β and activity of GSK3β. WT ESCs analysed for the expression of Pramel7 in presence of PD only showed an upregulation of Pramel7 (Fig. 27). Interestingly, DKO ESCs cultured in the same conditions showed 5-fold downregulation of Pramel7 (Fig. 28b) whereas the DKO_GSK3β and DKO_K85A ESCs carrying an active form of GSK3β and a kinase inactive GSK3β respectively showed slight

upregulation of Pramel7 (Fig. 28c & Fig. 28d). PD is a specific inhibitor of Erk kinase, which inhibits the phosphorylation of Erk. Erk activation promotes responsiveness to the differentiation cues. Upregulation of Pramel7 by inhibition of Erk in presence of GSK3β suggests the existence of a yet unidentified transcription factor regulated by these two kinases together, which is involved in this transcriptional circuitry. One possible candidate can be ESR1 (estrogen receptor alpha), which has binding sites on Pramel7 promoter. ESR1 belongs to a group of estrogen receptors localized in both cytoplasm and nucleus. It is a DNA binding transcription factor, which modulates gene and subsequently protein expression by activating or repressing gene transcription. It can recruit and bind to transcriptional coactivators and corepressors both in the presence and absence of hormone (Levin, 2005). ESR1 is activated upon phosphorylation of Ser118 by Erk and GSK3β blocks its transcriptional activity by inhibiting phosphorylation of nuclear ESR1 (Kato et al., 1995). Activation of PI3K/AKT pathway results in derepression of ESR1 by inhibiting the activity of GSK3β. This results in enhanced activity of ESR1 (Levin, 2005). Cardona-Gomez and colleagues have also shown that ESR1 forms a complex with β-catenin and GSK3β in the hippocampus and the presence of the estradiol results in the release of β-catenin from the complex thus promoting the transcriptional activity of β-catenin (Cardona-Gomez et al., 2004). However, this has not been shown in ESCs. One potential explanation to our finding that Pramel7 is upregulated in ESCs stimulated with PD only is that inhibition of Erk by PD and presence of GSK3β results in inactivation of ESR1, which can hypothetically be a transcriptional repressor of Pramel7. Taken together, the above findings confirm the importance of both presence and activity of GSK3β in the transcriptional regulation of Pramel7. Further experiments need to be performed to define the role of "the hypothetical transcriptional repressor- ESR1" in regulation of Pramel7.

Another question that still lingers is whether the GSK3β is involved in STAT3-dependent transcription of Pramel7. This can be either through direct interaction with STAT3 via phosphorylation of STAT3 or by forming a physical complex with it. Studies carried out by Beurel *et al* (2008) in mouse primary astrocytes, microglia and macrophages derived cells show that GSK3β is required for the recruitment of STAT3 to the receptors and promotes tyrosine phosphorylation (Beurel and Jope, 2008). Our finding from the western blot analysis on DKO and E14 ESCs do not show any changes in the phosphorylation of Stat3 thus suggesting that the role of GSK3β in recruitment of STAT3

to the receptor does not occur in ESCs. There is no physical interaction between the two proteins either as shown in Fig. 31.

Presence of leucine-rich repeat domain on Pramel7 protein suggests that it might act via protein-protein interactions. However, our immunoprecipitation studies do not show any physical interaction between GSK3β and Pramel7. Thus, the possible regulatory mechanism can be via direct interaction between GSK3β and another candidate, which subsequently regulates the expression of Pramel7 (Fig. 32).

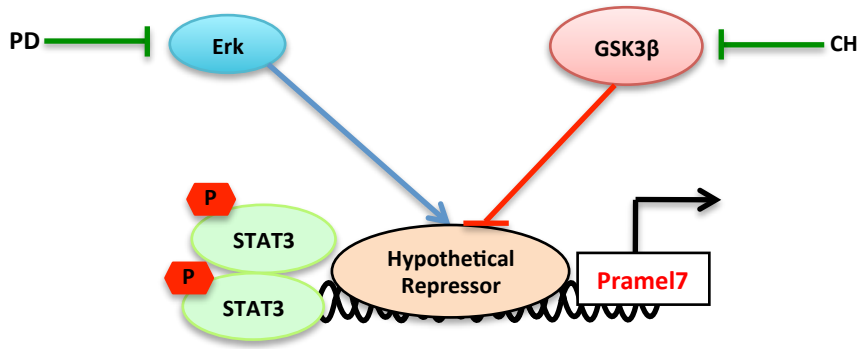

Figure 32: Hypothetical model regulating Pramel7 transcription

ESR1 (hypothetical repressor) has binding sites on Pramel7 promoter. Under normal conditions, Erk phosphorylates and activates ESR1 and GSK3ß blocks its transcriptional activity by inhibiting its phosphorylation. Hypothetically, inhibition of ESR1 in the presence of GSK3ß and repression of Erk using PD can be a possible mechanism regulating transcription of Pramel7.

D. Concluding Remarks and Future Directions

Embryonic stem cells were first established in 1981 from the inner cell mass of the mouse blastocyst. The remarkable property of pluripotency possessed by ESCs makes them a promising tool for regenerative medicine. Elucidating how these cells are established and propagated still remains a fascinating scientific challenge. A deeper understanding of the mechanisms that enable self-renewal of ESCs in a pluripotent state is necessary to realize their therapeutic potential. A detailed investigation will harness the methods for generating ESC lines and finding differentiation methods for human cells suitable for transplantation.

The rat is a widely used organism in the biomedical research. Given the importance of rat in physiological and pharmacological studies, the availability of authentic germline-competent ESCs offers the opportunity to knockin or knockout genes of choice by homologous recombination and produce genetically modified rats, which can be used to study human diseases. Understanding the mechanisms regulating pluripotency in rat ESCs is essential for generating transgenic rats and may help in finding genetic regulators that play an important role in both rat and human ESCs self-renewal and pluripotency processes. However, it is clear from our study on rat ESCs that while preliminary screening of established ESC lines for pluripotency markers, normal karyotype, pathogen-free status and differentiation potential is an essential quality control measure before proceeding with generation of chimeras to prove the authenticity of ESCs, there is still an unknown variable that plays an important role in determining whether a cell line is capable of being transmitted through germline. Hence, it would be of great importance to deeper understand how signaling mechanisms regulate ESCs self-renewal and differentiation in mice and then apply the knowledge gained to the rat ESCs. Although, some problems remain in the rat ESC field, recently devised techniques using zinc finger nucleases and transcription activator-like effector nucleases can target genes for producing knockout and knockin rats (presented in the chapter 4 of the introduction) without the use of ESCs. Unlike the ESC-based gene targeting, this technology fails to produce precise modifications for condition and inducible knockouts and is quite expensive. Nevertheless, multiple methods are being devised to target rat genes, which will largely eliminate the technical obstacles that prevented rat from being a genetic research system.

Regarding detailed understanding of the mechanisms regulating pluripotency of ESCs, it has been considered to depend on interaction between various important transcriptional circuitries, among which the most prominent one has been the activation of Stat3 through cytokine LIF. However, downstream targets of Stat3 have still remained elusive. Recently, we have shown that Pramel7 is a direct downstream target of Stat3 in the LIF/STAT3 pathway and plays an important role in murine ESCs. Concerning the second study reported here, experiments carried out on mESCs derived and expanded in 2i+LIF conditions clearly provide evidence for a potential role of GSK3β derepression and inhibition of Erk independent of LIF/STAT3 pathway in regulation of Pramel7. However, Pramel7 is not a direct functional target of GSK3β and hypothetically might be regulated by a transcription factor such as ESR1, which is a direct downstream target of both GSK3β and Erk. Future work is required to explore the mutual interaction between GSK3β, Erk and ESR1 in the regulation of Pramel7 transcription. It will also be of interest to carry out perturbation studies on ESR1 to demonstrate response of Pramel7. Through protein interaction studies, one can identify other targets of GSK3β and Erk that can modulate Pramel7 expression. This will be necessary for thorough understanding of the underlying mechanisms regulating pluripotency in mouse ESCs via Pramel7.

As it is known that Pramel7 plays a critical role in maintenance of pluripotency in mESCs, it will be interesting to functionally characterize its role in rat ESCs and examine whether the expression of Pramel7 can stabilize rat ESCs and lead to successful derivation of germline-competent lines from various strains. It is known that inner cell mass contains a heterogenous population of loosely committed cells which are differently balanced or biased in different species and strains. Thus, it will be of great interest to perform gene expression analysis on different rat strains for Pramel7. It is possible that robust germline competent strain like DA can retains its pluripotency due to higher expression of Pramel7. Lastly, it is not surprising that there is a difference in the preimplantation development of mouse and rat where, the mouse reaches the blastocyst stage at E3.5 and rat at E4.5. This difference highlights the complexity of molecular processes maintaining pluripotency in the two species. It will be of significance to compare the expression pattern of Pramel7 and other master genes both *in vivo* and *in vitro* between the two rodent species and eventually get closer to understanding mechanisms regulating self-renewal and pluripotency. This knowledge would promote the establishment of truly pluripotent stable ESC lines from species other than the mice, for instance the rat.

E. Materials and Methods

1. Project I: Establishment of pluripotent germline competent rat embryonic stem cells from Brown Norway strain

1.1. Reagents for ESC derivation

M2 medium (Sigma, M7167)

Acid tyrode's solution (Sigma, T1788)

Anti-rat whole serum (Sigma, R5256)

Rat complement serum (self-made)

1.2. Cell culture reagents

Media and Supplements

N2B27	1:1 (below media)
	Neurobasal (Gibco, 21103-049): DMEM:F-12 (Gibco, 21331-020)
	Penicillin-Streptomycin-L-Glutamine, 2mM (Gibco, 25030-024)
	N2, 1:200 (Gibco, 17502- 048)
	B27, 1:100 (Gibco, 17504-044)
	50mM 2-mercaptoethanol (Gibco, 21985-023)
2i	N2B27 supplemented with:
	PD0325901, 1µM
	CHIR99021, 3µM

Embryoid Body formation

Neural Differentiation	DMEM: F-12 (Gibco, 21331-020)
	N2, 1:100(Gibco, 17502- 048)
	B27, 1:50 (Gibco, 17504-044)
	Penicillin-Streptomycin-L-Glutamine, 2mM (Gibco, 25030-024)
Smooth Muscle Differentiation	DMEM
	10% Fetal calf serum

1.3. Cytokines and Inhibitors:

Leukemia Inhibitory factor (LIF): 1000U/ml (Millipore, ESG1107)

Inhibitors PD0325901, 1μM (Stemgent, 04-0006)

CHIR99021, 3μM (Stemgent, 04-0004)

1.4. Other tissue culture reagents

Gelatin (0.1% in distilled water, autoclaved)

Laminin, 10ug/ml (Sigma, L2020)

Accutase (1x)

PBS (PAA)

1.5. Derivation of ESCs lines from Brown Norway rats

Brown Norway (BN) male and female rats were obtained from JANVIER SAS and used for derivation of rat ESC lines. 6 to 12 week old BN females were checked for estrus by using estrus cycle monitor EC40 as previously described (Ramos S D et al 2004). Wild type BN males were mated with wild type female ones for 24hrs and then housed separately. Rat blastocysts at E4.5 days post coitum (dpc) were collected by flushing the uterus with the M2 medium. They were treated briefly with acid tyrode's solution to remove zona pellucidae. The blastocysts without the zonae were then incubated with at 37°C in 20% anti-rat whole serum for 3 hrs. Later, they were washed in N2B27 and incubated for 15-20 mins in rat serum as a source of complement. The lysed trophectoderm was then removed by pipetting and the isolated ICMs were transferred to a 48-well plate containing N2B27+2i+LIF on SNL feeders at 37°C in an incubator with 5% CO2. On day 5, outgrowths of the ICMs were washed and then individually dissociated into single cell suspension using Accutase, spun down and resuspended in required amount of N2B27+2i+LIF and transferred to a 24-well plate containing SNL feeders. ESCs were passaged every 48-72 hrs.

Karyotyping of rat ESCs

Rat ESC lines were cultured in 10cm laminin coated dishes for 48 hrs and until 70% confluent. The cells were then incubated in 0.01mg/ml colcemid for 3hrs at 37°C. Cells were then washed with PBS, disaggregated with Accutase and then pelleted by centrifugation at 1000rpm for 4 mins. After removing the supernatant, they were then resuspended in 10ml of pre-warmed hypotonic solution (0.56% KCl) and incubated for 20 mins in water bath. A few drops of freshly made fixative consisting of methanol and

acetic acid (3:1 ratio) was added and cells were centrifuged at 1200rpm for 5 mins. After 2 more rounds of fixation step carried out by resuspending the cells in 10ml of fixative, the pelleted ESCs were resuspended in 2ml of fixative at the final step. Chromosome spreads were done by dropping the cell solution on the slides from approximately 1m height and the karyotype analysis was performed using the Giemsa stain.

1.6. *In vitro* characterization of rat ESCs

1.6.1. Expression of pluripotency factors

The established ESC lines were screened for the expression of pluripotency factors, Oct4 and SSEA-1, by immunofluorescence staining. The rat ESC lines were cultured in 35mm culture dishes until 70% confluents. Cells were fixed in 4% PFA for 15 minutes, blocked and permeabilized in PBS, 0.1% Triton x100, 3% Horse serum. Primary antibodies were incubated in the same buffer overnight at 4°C. Secondary antibodies were incubated for 1hr at RT. Plates were washed 3 x 15mins in PBS after primary and secondary antibody incubations. Nuclei were stained with DAPI.

1.6.2. Alkaline phosphatase staining

Reagents

-10x Alkaline Phosphatase Buffer without Mg

- 1M Tris-HCl
- 1M NaCl

  Adjust the pH to 9.5 and autoclave the solution

-1xAP Buffer with $MgCl_2$ = AP Buffer

For a 50ml Aliquot: 5ml 10x AP Buffer, 2.5ml 1M $MgCl_2$, 42.5ml ddH_2O

-Staining solution

- AP buffer
- 0.5µl/ml NBT (nitro-blue tetrazolium chloride)
- 3.5µl/ml BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt)

-Tris-EDTA

- 20mM Tris-HCl
- 5mM EDTA
- H_2O

ESCs were cultured on 35mm dishes prior to staining for Alkaline phosphatase. Adherent rat ESCs were once washed with PBS and fixed in 4%PFA for 15 minutes. Cells

were then washed twice with AP buffer for 10 mins. Staining solution was added to the dishes and these dishes were places in dark for 30-60 mins. The intensity of the staining was checked and later, the reaction was blocked by adding 1ml of Tris-EDTA. The plates were then washed with PBS thrice for 10 mins and stored in 2ml of PBS at 4°C.

1.6.3. Expression of differentiation markers

For embryoid body formation, 1-3 million cells were plated on low attachment 10cm bacterial plates with embryoid body medium (EB). Medium was changed every 48 hrs for the next 10 days.

For monolayer differentiation, 10-50 thousand rat ESCs were plated on a laminin-coated 6-well dishes with neural or smooth muscle differentiation medium. They were cultured for 10 days. Medium was changed every 24 hrs to get rid of dead cells. They were then fixed with 4%PFA and stained for differentiation markers.

Table 8: List of Antibodies I

	Antibody	Dilution	Source	Catalogue number
Primary	Oct3/4	200	Santa Cruz Biotechnology	SC-9081
	SSEA-1	500	Santa Cruz Biotechnology	SC-101462
	β-III Tubulin	500	Sigma Aldrich	T8660
	PAX6			
	Smooth muscle actin	500	Sigma Aldrich	A2547
	β-catenin	500	BD Transduction Laboratories	610154
Secondary	Alexa fluor goat anti-rabbit 594	500	Invitrogen	A11037
	Alexa fluor goat anti-mouse 488	500	Invitrogen	A11029

1.7. In vivo characterization of rat ESCs

1.7.1. Teratoma formation

$1x10^6$ cells were injected subcutaneously into each dorsal flank of NOD/SCID mice. Three weeks after injection, teratomas were collected and fixed in 4% paraformaldehyde. They were then embedded in paraffin wax, sectioned and immunostained for β-III Tubulin, Smooth muscle actin and β-catenin.

1.7.2. Generation of chimera

The ESCs lines were used for generating chimeric animals via blastocyst injection. Lewis and Wistar rats were used as the donor and foster rats respectively and were obtained from JANVIER SAS.

Prior to the injections, ESCs were cultured in N2B27+2i+LIF for atleast 3 or more passages to ensure full recovery from any stress caused in the process of cryopreservation. On the day of injection, the rat ESC cells were dissociated using Accutase and the centrifuged pellet was resuspended in N2B27+2i+LIF to separate the feeders from the ESCs. After the separation, the ESCs were centrifuged again and resuspended in N2B27+2i+LIF+HEPES and incubated on ice. Donor blastocysts were collected at E4.5 dpc from pregnant Lewis females that were mated with Lewis males. For blastocyst injection, 10-12 rat ESCs were injected into single blastocysts using a beveled Transfertip. Injected blastocyst were incubated at 37°C for atleast an hour and then approximately 10-12 blastocysts were transferred into the uterine horns of day3.5 pseudo-pregnant Wistar female. Chimerism in the offsprings was identified by coat color chimerism, detected by the presence of non-agouti brown hairs against an agouti coat color background.

2. Project II: Elucidating the molecular mechanisms regulating transcription of Pramel7 in mouse embryonic stem cells

2.1. Cell culture reagents

2.1.1. Cell culture media

GMEM Complete	Glasgow Minimal Essential Medium (Sigma, G5154)
	Fetal Calf Serum (10%)
	Non-essential amino acids (Gibco, 11140-035)
	L-Glutamine, 2mM (Gibco, 25030-024)
	Sodium Pyruvate, 1mM (Gibco, 11360-039)
	50mM 2-mercaptoethanol (Gibco, 21985-023)
	LIF (1000U/ml)
N2B27	1:1 (below media)
	Neurobasal (Gibco, 21103-049): DMEM:F-12 (Gibco, 21331-020)
	Penicillin-Streptomycin-L-Glutamine, 2mM (Gibco, 25030-024)
	N2, 1:200 (Gibco, 17502- 048)
	B27, 1:100 (Gibco, 17504-044)
	50mM 2-mercaptoethanol (Gibco, 21985-023)
2i	N2B27 supplemented with:
	PD0325901, 1μM (Stemgent, 04-0006)
	CHIR99021, 3μM (Stemgent, 04-0004)
EB formation	Glasgow Minimal Essential Medium (GMEM)
	Fetal Calf Serum (10%)
	50mM 2-mercaptoethanol (Gibco, 21985-023)
	Non-essential amino acids (Gibco, 11140-035)
	Penicillin-Streptomycin-L-Glutamine, 2mM (Gibco, 25030-024)
	Sodium Pyruvate, 1mM (Gibco, 11360-039)

Neural Differentiation	DMEM: F-12 (Gibco, 21331-020) N2, 1:100(Gibco, 17502- 048) B27, 1:50 (Gibco, 17504-044) Penicillin-Streptomycin-L-Glutamine, 2mM (Gibco, 25030-024)
Smooth Muscle Differentiation	DMEM (PAA, E15-810) 10% Fetal calf serum

Cytokines and Inhibitors:

Leukemia Inhibitory factor (LIF): 1000U/ml (Millipore, ESG1107)

Inhibitors	PD0325901, 1µM (Stemgent, 04-0006) CHIR99021, 3µM (Stemgent, 04-0004) LY294002, 5µM

2.1.2. Other tissue culture reagents

Antibiotics	Puromycin (Sigma, P9620) Hygrogold B (Invivogen,): 150µg/ml, 100µg/ml serum-free Geniticin (Invitrogen,): 1mg/ml serum free

Gelatin (0.1% in distilled water, autoclaved)

Trypsin_EDTA (1x)

PBS (PAA)

2.1.3. Embryonic Stem Cell lines

- β-catenin knockout
- β-catenin N-terminal mutant (D164A)
- β-catenin C-terminal mutant (delta C)
- Wild type

Heterozygous mice obtained from Prof. Basler's Laboratory. Mouse ESCs derived and cultured in house in N2B27+2I+LIF

- Wild type
- STAT3 knockout

mES cells derived from 129 mice cultured in N2B27+2I+LIF obtained from Prof. Austin Smith's laboratory

- GSK3 double knockout DKO: ES cells with targeted knockout of both alleles of *Gsk3-α* and *–β*.
- DKO_GKS3β: DKO ES cells targeted with knock-in of wild type GKS3β. Cultured in ES medium with hygrogold and LIF
- DKO_K85A: DKO ES cells targeted with knock-in of kinase inactive GKS3β. Cultured in ES medium with hygrogold and LIF

Obtained from Bradley Doble (Kelly et al., 2011)

- WT_S33Y

  Wild type ESCs with 129 background stably expressing constitutively active β-catenin due to single point mutation in the phosphorylation site and carrying neomycin resistance.

2.2. Cell culture

2.2.1. Tissue culture routine

All cell lines were maintained in 37C, humidified incubators maintained at 5% CO2.

2.2.2. Generation and derivation of mouse ESCs

E2.5 embryos were isolated from intercrossing heterozygous mice for the mutants. They were then incubated in KSOM+2I for a day after which they were transferred to N2B27+2I for two days. At E4.5, most embryos had hatched from the zona pellucida (ZP) and the ones with intact ZP were treated with Acid tyrode solution to get rid of it. Embryos were then incubated with 10% anti-mouse serum for a few hours before washing them in N2B27 and transferring them to 10% complement sera (rat serum) for 10-15 mins to lyse the trophectoderm cells. The embryos were transferred to N2B27 for an hour and the trophectoderm was later separated from inner cell mass (ICM) by mouth-pipetting for each embryo. The cleaned ICM was then transferred to gelatin coated 48-well dish to grow for 4-5 days in N2B27+2I+LIF. After 4-5 days, the ICM was dissociated using 0.5% Trypsin-EDTA and the cells were plated on a new gelatin coated 48-well dish. After establishment of mESCs, routine serum-free culture was performed using N2B27 supplemented with 2I and LIF. Media was changed every two days and cells were passaged when approaching confluency. To passage, cells were washed in phosphate buffered saline (PBS), incubated with 0.5 x trypsin-EDTA for approximately 3 minutes or until cells detached, resuspended in 5-10 trypsin volumes of media, harvested by centrifugation (1000rpm, 4mins) and replated at the required density.

2.2.3. *In vitro* differentiation of mESCs

For embryoid body differentiation, 1-3 million mESCs were plated on 10cm low-attachment bacterial plates and cultured in EB medium for 10 days. Media was changed daily. EBs were washed, centrifuged and collected after 10 days in RLT buffer with 2-mercaptoethanol for RNA extraction and real-time PCR analysis. For monolayer differentiation, 10-50 thousand ESCs were plated on gelatin coated 6-well dish and cultured with either neural or smooth muscle differentiation media for 10 days.

2.3. Manipulation of Cells

2.3.1. Stable transfection- Electroporation

In order to stably integrate transgene into mESCs, 8μg of β-catenin S33Y plasmid encoding the single point mutation in β-catenin was linearized by restriction digest (enzyme Ssp1), purified by DNA purification kit and electroporated into cells. 5 million cells per transfection were harvested by trypsinisation, washed in PBS, resuspended in 800μL PBS and placed in an electroporation cuvette (Biorad, 165-2081EDU). 10μg linearized DNA was added to the cuvette. Cells were then electroporated (Cap 3.0μF, 0.8kV) and transferred to 9.2ml pre-warmed N2B27+2I+LIF medium. Cells were then plated in gelatinized 6cm tissue-culture dishes at 2 million cells per dish in N2B27+2I+LIF. 24 hours later the appropriate selective drug, neomycin was added. Cells were cultured for a further 9-11 days, changing media every 2 days. Once colonies had reached a suitable size, isolated colonies were 'picked' and transferred to gelatine-coated 96-well tissue culture plates. This was considered passage 1 for newly-derived transgenic lines. Lines were expanded by passaging to multi-well plates of increasing well size until sufficient cells were obtained to freeze down, typically a 6-well plate.

2.3.2. Transient transfection – Lipofection for siRNA knockdown for GSK3β

siRNAs were transfected at a final concentration of 20 nM using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. Embryonic stem cells were transfected and maintained in 2i + LIF for a further 48h. Gsk3α, Gsk3β and control siRNAs were obtained from Qiagen (GS606496, GS56637 and SI03650325, respectively).

2.4. Molecular Biology

2.4.1. DNA extraction and genotyping for mESCs

mESCs pellets were collected and digested with 50μl of DNA lysis buffer (100mM Tris HCl, ph 7.5; 5mM EDTA; 100mM NaCl; 0.2% SDS; 1mg/ml proteinase K added just before use) at 55C overnight. The samples were then heat inactivated at 95C for 15 minutes and stored at -20C for long -term storage. To test for the presence of wild type and mutant alleles in the ESCs derived, genomic PCR was carried out. The PCR mix, primers and program are given below.

PCR mix:

Genomic DNA	2µL
10x Buffer	2µL
Q solution	4 µL
MgCl2	0.8 µL
Primers (10µM)	1µL each
dNTPs (10mM)	0.5µL
Taq polymerase	0.5µL
dH2O	8.2µL

PCR conditions for β-catenin KO ESCs

95C- 2'

35 cycles

95C- 30"

56C- 40"

72C- 55"

72C- 5'

4C- ∞

Primers for β-catenin KO and WT genotypes

RM41, RM42 and RM43 (Brault et al., 2001)

PCR condition for β-catenin D164A and DC mutants

95C- 2'

35 cycles

95C- 30"

58C- 40"

72C- 55"

72C- 5'

4C- ∞

Primers for D164A and Δ C mutant

D164A: 5301.1 and 5301.2 (Valenta et al., 2011)

DC: 5301.3 and 5301.4 (Valenta et al., 2011)

2.4.2. Immunofluroscence

Cells were fixed in 4% PFA for 15 minutes, blocked and permeablised in PBS, 0.1% Triton x100, 3% Horse Serum, 1% BSA. Primary antibodies were incubated in the same buffer overnight at 4C. Secondary antibodies were incubated for 1hr at RT. Plates were washed 3 x 15mins in PBS after primary and secondary antibody incubations. Nuclei were stained with DAPI. The table below lists the different antibodies used.

Table 9: List of Antibodies for immunofluorescence

	Antibody	Dilution	Source	Catalogue number
Primary	Oct3/4	200	Santa Cruz Biotechnology	SC-9081
	SSEA-1	500	Santa Cruz Biotechnology	SC-101462
	β-III Tubulin	500	Sigma Aldrich	T8660
	Smooth muscle actin	500	Sigma Aldrich	A2547
Secondary	Alexa fluor goat anti-rabbit 594	500	Invitrogen	A11037
	Alexa fluor goat anti-mouse 488	500	Invitrogen	A11029

2.4.3. RNA extraction

2-3 million cells were harvested by trypsinisation, washed in PBS and lysed in buffer RLT (Qiagen RNeasy kit). Lysates were stored at -80C or used directly for RNA extraction. Lysates were homogenized by spinning through a Qiashredder (Qiagen, 79654) and subjected to RNA extraction using the RNeasy Kit (Qiagen, 74104) according to the manufacturers protocol and incorporating an on-column DNase digest (Qiagen, 79254) to remove genomic DNA. Eluted RNA concentration was determined by OD measurement using Nanodrop 1000 spectrometer. Resuspended RNA was stored at -80C.

2.4.4. cDNA synthesis

cDNA was synthesized using the Invitrogen Superscript III First-Strand Synthesis Kit (Invitrogen, 11752050) according to the manufacturer's protocol using oligo-dT primers. 0.5-1μg RNA was used as template. cDNA was diluted 1:10 in sterile water and used for qRT-PCR.

2.4.5. qRT-PCR

Real-time quantitative PCR was performed using the Applied Biosystems machine. Sybr Green based qRT-PCR was performed. Reactions were performed in 10μL reaction volumes using the Sybr Green Master Mix (Qiagen,) in triplicates. Values were

calculated using the comparative threshold cycle (ΔCt) method. See the table below for the primers used.

Master mix per reaction:

cDNA: 2µl

Sybr green Master mix: 5µl

Primer (10µM): 0.5µl

PCR grade water: 2µl

Table 10: Sybr Green Real-Time PCR primers

Gene	Forward primer	Reverse primer
Oct3/4	5'-ggc gtt cgc ttt gga aag gtg ttc -3'	5'-ctc gaa cca cat cct tct ct -3'
Nanog	5'-aca agg gtc tgc tac tga gat gc-3'	5'-gga gac ttc ttg cat ctg ctg g-3'
Rex1	5'-aga aag cag gat cgc ctc ac-3'	5'-agg gaa ctc gct tcc aga ac-3'
Pramel7	5'-gag gag aag cag aac atc agc aag a-3'	5'-ctc tta gag gcg tga cat cta ggt t-3'
STAT3	5'-ggc aag ggc ttc tcc ttc tg-3'	5'-agc tgc tgc ttg ttg gtg tat gg-3'
Axin2	5'-ggg gga aaa cac agc tta ca-3'	5'-act ggg tcg ctt ttg aa- 3'
CyclinD1	5'-gcc atc caa act gag gaa aa-3'	5'-tca cct ctt ccc tca cat cc-3'
B-catenin	5'-gtg caa ttc ctg agc tga ca- 3'	5'-ctt aaa gat ggc cag caa gc-3'
FGF5	5'-aaa gtc aat ggc tcc cac gaa-3'	5'-ctt cag tct gta ctt cac tgg-3'
Sox9	5'-aca cac ttt cgt gga ggc gta ga-3'	5'- acc agg ggc cac tgt cag atg t -3'
GATA4	5'-gcc tgt atg taa tgc ctg cg- 3'	5'-ccg agc agg aat ttg aag agg -3'
T-brachyury	5'-atg cca aag aaa gaa acg ac-3'	5'-aga ggc tgt aga aca tga tt-3'
GSK3β	5'-tcc att cct ttg gaa tct gc -3'	5'-caa ttc agc caa cac aca cag c -3'
β-actin	5'-cat cca ggc tgt gct gtc cct gta tgc-3'	5'-gat ctt cat ggt gct agg agc cag agc-3'

2.4.6. Western blot analysis

Cultured mESCs were scraped and resuspended in 200µl of RIPA buffer (containing--- Protease inhibitors was added freshly before use.) Samples were then centrifuged for 3 minutes at full speed at 4C. The supernatant was transferred to a new tube and protein concentration was determined using the BCA protein assay kit. 10µg of protein extract was used for Western blot analysis. Samples were subjected to SDS-PAGE and blotted onto PVDF membrane (Millipore, Volketswil, Switzerland) at 100 V for 1.5h at 4°C. Immunodetection and chemiluminescent visualization were performed as recommended by the supplier of the chemiluminescence blotting kit (Roche Diagnostics, Rotkreuz, Switzerland).

2.4.7. Immunoprecipitation

mES cells were grown in 10cm dishes in the appropriate conditions. Cells were washed once in cold PBS. Lysates were collected by scraping the plates with a cell scraper (Greiner) and pippeting the lysate into 1.5ml eppendorfs on ice. They were then either stored at -80C or processed through sonication and then frozen with liquid N2.

25μL Flag or HA bead slurry per sample was placed in 1.5ml eppendorfs, centrifuged, supernatant removed and beads washed in 1ml PBS 2-3 times and then with NE buffer. 50ul of sample was removed as an input sample. Bradford assay was carried to measure the protein lysate concentration and 1mg of it was added to the beads containing antibody. The tubes were tumbled overnight at 4C, beads pulled down by centrifugation, supernatant removed and beads washed in 1ml cold NE buffer 3 times. On the last wash, all supernatant was removed, 14μL of sample buffer and reducing agent mix added and samples boiled for 5mins. Lysates can be stored at -20C or used directly for Western blot analysis. See table 4 for details of antibodies and concentration.

Table 11: List of Antibodies and concentration for Western Blot and Immunoprecipitation

	Antibody	Dilution	Source	Catalogue number
Primary	β -catenin	5000	BD transduction laboratories	610154
	Anti-GSK3α/β	1000	Invitrogen	44610
	Total GSK3	1000	Cell signalling technology	5676P
	Phospho STAT3 (Y705)	1000	Cell signalling technology	9131S
	Phospho STAT3 (S727)	1000	Cell signalling technology	9134S
	Total STAT3	2000	Santa Cruz Biotechnology	SC482 (C-20)
	Anti-tubulin	10000	Sigma Aldrich	T9026
	Anti-Flag			
Secondary	Goat anti-rabbit HRP	2000-5000	Santa Cruz Biotechnology	SC2030
	Goat anti-mouse HRP	2000-5000	Santa Cruz Biotechnology	SC2031

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Annex

STEM CELLS®

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

Pramel7 Mediates LIF/STAT3-Dependent Self-Renewal in Embryonic Stem Cells

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Key Words. Embryonic stem cells • Differentiation • LIF • STAT3 • Self-renewal

ABSTRACT

A unique and complex signaling network allows ESCs to undergo extended proliferation in vitro, while maintaining their capacity for multilineage differentiation. Genuine ESC identity can only be maintained when both self-renewal and suppression of differentiation are active and balanced. Here, we identify Pramel7 (preferentially expressed antigen in melanoma-like 7) as a novel factor crucial for maintenance of pluripotency and leukemia inhibitory factor (LIF)-mediated self-renewal in ESCs. In vivo, Pramel7 expression was exclusively found in the pluripotent pools of cells, namely, the central part of the morula and the inner cell mass of the blastocyst. Ablation of Pramel7 induced ESC differentiation, whereas its overexpression was sufficient to support long-term self-renewal in the absence of exogenous LIF. Furthermore, Pramel7 overexpression suppressed differentiation in ESCs in vitro and in vivo. This process was reversible, as on transgene excision cells reverted to a LIF-dependent state and regained their capacity to participate in the formation of chimeric mice. Molecularly, LIF directly controls Pramel7 expression, involving both STAT3-dependent transcriptional regulation and PI3K-dependent phosphorylation of glycogen synthase kinase 3β. Pramel7 expression in turn confers constitutive self-renewal and prevents differentiation through inactivation of extracellular signal-regulated kinase phosphorylation. Accordingly, knockdown of Pramel7 promotes ESC differentiation in presence of LIF and even on forced STAT3-activation. Thus, Pramel7 represents a central and essential factor in the signaling network regulating pluripotency and self-renewal in ESCs. STEM CELLS *2011;29:474–485*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

ESCs are derived from the inner cell mass (ICM) of blastocyst [1, 2] and represent an important tool for the study of early embryonic development and the pluripotent state, mostly because of their two distinctive properties, that is, their ability to undergo indefinite mitotic self-renewal and to differentiate into a range of specialized cell types. A tightly balanced interplay between different pathways is necessary to promote self-renewal in ESCs. Some of these pathways act through the repression of factors that initiate differentiation programs [3–7]. STAT3, OCT3/4, SOX2, and Nanog are transcription factors that regulate various aspects of ESC fate and safeguard the maintenance of the pluripotent state [3, 5].

Derivation and maintenance of murine ESCs were originally achieved by using feeders or the cytokine leukemia inhibitory factor (LIF) in combination with fetal calf serum or the growth factor bone morphogenetic protein. LIF acts through the leukemia inhibitory factor receptor/gp130 complex to maintain pluripotency [8, 9]. Cultivation of ESCs on *Lif*-deficient fibroblasts leads to differentiation, indicating that they mostly provide LIF [10]. LIF-independent maintenance of mouse ESCs with retention of pluripotency (adult chimerism) has been previously described for cell lines, which overexpressed Nanog or KLF2 [11, 12]. In vitro, the overexpression of PEM/RHOX5 also maintains pluripotency without LIF, even though contribution to chimera has not yet been proven [13, 14]. Nowadays, it is possible to bypass LIF, feeders, and serum requirement by using two inhibitors (2i conditions) which block mitogen-activated protein kinase (extracellular signal-regulated kinases [ERKs]) and glycogen synthase kinase 3β (GSK3β) [15]. Interestingly, maximal self-renewal is obtained by combination of LIF and 2i confirming LIF/STAT3 signaling as an essential component of self-renewal in ESCs. Despite the importance of this pathway, STAT3 downstream target genes have remained elusive. In a recently performed microarray study, we found Pramel6 and Pramel7 (preferentially expressed antigen in ESCs upon conditional in melanoma like 6 and 7) strongly upregulated in ESCs upon conditional overexpression of STAT3 [14]. These findings indicate a potential role of these genes in the stabilization of ESCs. In this work, we aimed at the functional characterization of Pramel6 and particularly of Pramel7 in ESCs.

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Our results demonstrate that *Pramel7* is a new direct STAT3 target gene, fundamental for the LIF-mediated maintenance of pluripotency and for the inhibition of differentiation.

Materials and Methods

Cell Culture

Medium for E14 129/Ola and transgenic ESCs (complete medium [CM]-medium): Glasgow minimal essential (Sigma, Buchs, Switzerland, www.sigmaaldrich.com/switzerland-schweiz.html), 100 mM sodium pyruvate (Sigma), 10% fetal bovine serum (Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com), 50 mM β-mercaptoethanol (Gibco), 1× minimal essential medium nonessential amino acids (Gibco), 200 mM L-glutamine. Medium supplemented or not with 1,000 U/ml ESGRO murine LIF (Millipore, Chemikon, Zug, Switzerland, www.millipore.com). N2B27-medium: DMEM/F-12 (Invitrogen), Neurobasal (Invitrogen), 50 mM β-mercaptoethanol (Gibco), 200 mM L-glutamine, N2-Supplement 100× (Invitrogen), B27-Supplement 50× (Invitrogen). Medium supplemented with two inhibitors: 3 μM CHIR99021 (Stemgent, Cambridge, MA, www.stemgent.com) and 1 μM PD0325901 (Stemgent). *Lif* knockout fibroblast: *Lif* +/− mice [16] were mated and at E14.5 the fetuses were isolated. Heads and placentas were used for genotyping, whereas the rest of the embryo was trypsinized and cultured. *Lif* −/− fibroblasts were expanded and treated with mitomycin-C (10 μg/μl). Neural-differentiation medium: DMEM/F-12 (Invitrogen), B27-Supplement 50× (Invitrogen), N2-Supplement 100× (Invitrogen). For PI3K inhibition, 5 μM LY294002 were added to the N2B27 medium not supplemented with CHIR99021 and PD0325901.

Immunohistochemical Analyses

E14, transgenic, and recombined ESCs were cultivated for 11 days with CM-medium with or without LIF on *Lif* knockout feeders. At day 11, all cell lines were analyzed for OCT3/4 and stage-specific embryonic antigen-1 (SSEA-1) expression. For the serum- and feeder-free experiment, the same cells were cultivated on gelatinized plates for 9 days. N2B27 medium was supplemented with either both or one inhibitor or only LIF. ESCs were fixed in 4% paraformaldehyde and incubated overnight at 4°C with primary antibodies against OCT3/4 (rabbit anti OCT3/4, Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com) and SSEA-1 (Mouse mAb, Millipore). Secondary fluorescence-labeled antibodies were used for detection (anti rabbit Alexa Fluor 594 and anti mouse Alexa Fluor 488, Molecular Probes, Invitrogen). Nuclei of the cells were counterstained with DAPI (Roche, Basel, Switzerland, www.roche.ch).

Pramel7 Knockdown

shRNA vectors: four specific shRNA vectors against Pramel7 cloned in pGFP-V-RS vector (Origene, Rockville, MD, www.origene.com) and one negative control shRNA pGFP-V-RS vector. Sequences of shRNA are listed in Supporting Information Table 2. ESCs were transiently lipofected with either the shRNA constructs against Pramel7 or with the control vector by using FuGENE HD Transfection Reagent (Roche) and selected with puromycin. Transfection efficiency was monitored by EGFP fluorescence and Pramel7 knockdown was analyzed by real-time polymerase chain reaction (PCR). Both E14 wild-type (wt) and STAT3MER transgenic cells were cultivated on feeders with CM-medium either supplemented with LIF (E14 cells) or with hydroxy-tamoxifen (OHT; STAT3MER cells). STAT3MER cells were analyzed for alkaline phosphatase (AP) expression at day 8, respectively 4 of the knockdown. For STAT3MER cells, all the AP-positive colonies present in the 35-mm dishes were counted. For E14 cells, colonies with greater than 80% staining were classified as "undifferentiated," 20%–80% staining as "mixed," and less than 20% as "differentiated."

LIF Induction Experiment

wt ESCs, established under feeder- and serum-free conditions, were cultivated on gelatinized 35-mm dishes in N2B27 + 2i medium. Cells were then incubated for 4 hours in N2B27 + 2i, N2B27 + PD0325901, N2B27 + CHIR99021, N2B27 + LY294002 or in N2B27 without inhibitors. After 4-hour incubation, LIF was added to the media. Total RNA was extracted after 30 minutes, 1 hour, 3 hours, and 5 hours of LIF incubation. Reverse transcription and real-time PCR were performed.

Part of Material and Methods is provided in the Supporting Information.

Results

Characterization of *Pramel6* and *Pramel7* Genes

In the mouse, *Pramel6* and *Pramel7* cluster on chromosome 2(D) in opposite orientations (Supporting Information Fig. 1A). The amino acid composition of both proteins is very similar (Supporting Information Fig. 1B). Search for recognizable domains in the open reading frame of both genes using SMART (http://smart.embl-heidelberg.de) and myHits (http://myhits.isb-sib.ch/cgi-bin/motif_scan) revealed the presence of conserved leucine-rich repeats (LRRs), which usually participate in protein-protein interactions. The presence of these types of domains and the absence of conserved domains typical for transcriptional factors suggests that the *Pramel* family might not directly regulate gene transcription but rather act via protein-protein interaction.

Pramel6 and Pramel7 Expression Is Restricted to the Late Preimplantation and is Silenced in the Early Postimplantation Embryonic Stages

Whole mount in situ hybridization studies in the preimplantation embryos showed a homogeneous expression of Pramel6 in all cells of the morula and the blastocyst, whereas Pramel7 mRNA distribution was restricted to the interior part of the morula and the ICM of the blastocyst (Supporting Information Fig. 1C). Gene expression analyses of early postimplantation embryos (E6.5) revealed that at this developmental stage *Pramel6* and *Pramel7* genes are silenced (Fig. 1A). At this stage, the embryos expressed, as expected, both Nanog and DPPA3. The Nanog expression was restricted to the embryos, whereas DPPA3 was detected in the embryo as well as in the decidua (Fig. 1A).

In vitro, *Pramel6* and *Pramel7* are constitutively expressed in ESCs (Fig. 1B) independent of whether the cells are cultivated with serum, feeders, and LIF, or with N2B27 + 2i (serum free, feeder free, and LIF free). Expression of both genes disappears as soon as the cells differentiate. In accordance with our previous results [14], the presence of LIF in the medium resulted in an increased expression of both genes, presumably because of a direct activation of STAT3 (Fig. 1B).

Taken together, these observations suggest a possible role of both *Pramel6* and *Pramel7* in the maintenance of pluripotency in vivo as well as in vitro.

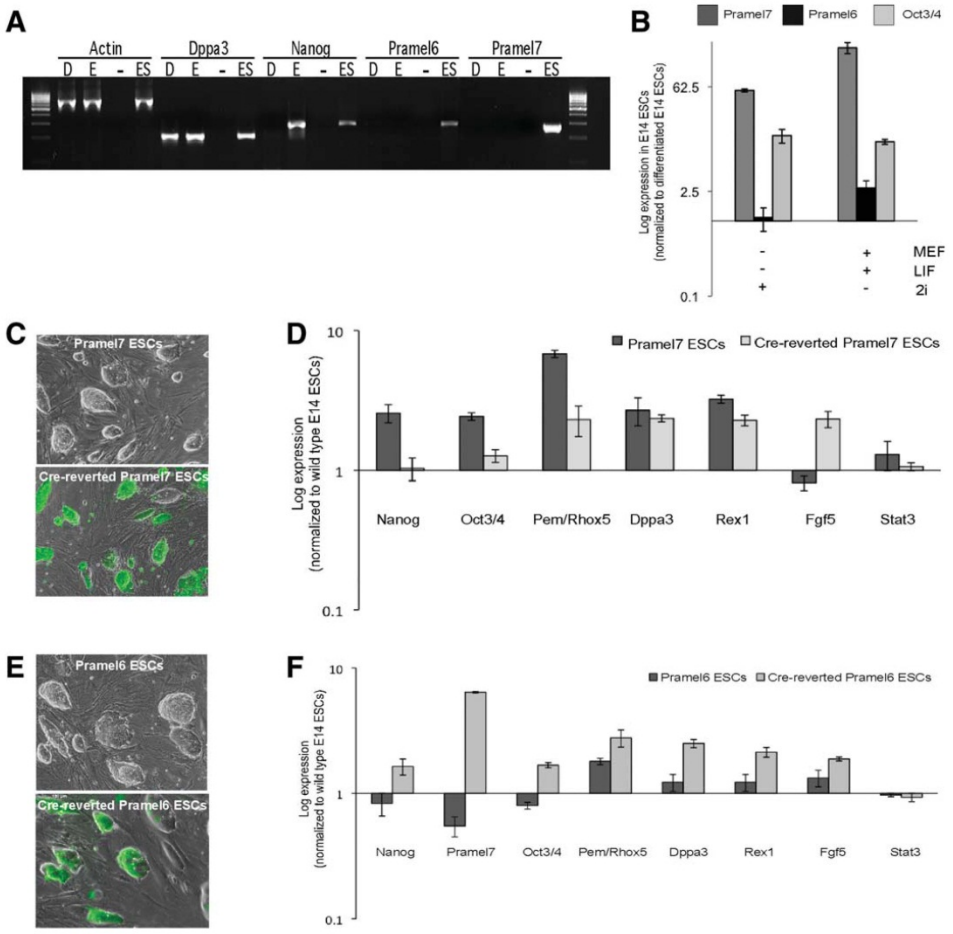


Figure 1. Endogenous expression of Pramel6 and Pramel7 and characterization of the transgenic clones. **(A):** Expression of Pramel6, Pramel7, Dppa3, and Nanog in early postimplantation embryos (E6.5). Pramel6 and Pramel7 expressions are absent in both decidual and embryonic tissue but present in ESCs. Negative control (−). **(B):** Real-time polymerase chain reaction (PCR) analysis of wild-type (wt) ESCs cultivated in presence of LIF and feeders (MEF) or in presence of the extracellular signal-regulated kinases and glycogen synthase kinase 3 inhibitors (2i). Expression levels of Pramel7, Pramel6, and Oct3/4 are compared with differentiated ESCs. **(C, E):** Representative morphology of Pramel7 and Pramel6 overexpressing cells and Cre-reverted (EGFP expressing) ESCs cultivated in presence of LIF and feeders. **(D, F):** Real-time PCR analyses of known pluripotency-related genes in Pramel7, Pramel6, and in Cre-reverted ESCs. Data were normalized to the expression level in the wt ESCs. Abbreviations: D, decidual tissue; E, embryonic tissue; ES, ESC; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblasts.

Pramel7 but Not Pramel6 Overexpression in ESCs Induces General Upregulation of Known Pluripotency-Related Genes

We generated ESCs conditionally overexpressing LoxP-flanked open reading frames of *Pramel6* or *Pramel7*, which can be excised by Cre-recombinase simultaneously bringing *egfp* under the CAG promoter (Supporting Information Fig. 2A). Overexpression experiments were performed in E14 129/Ola (E14) ESCs and, if not specified, they were cultivated in presence of feeders and serum (referred as CM). All clones showed a high expression of the transgene, which reverted to wt levels once recombined (Supporting Information Fig. 2B and 2C). All transgenic clones showed the classical morphology of pluripotent ESCs (Fig. 1C, 1E, and Supporting Information Fig. 2E) and expressed the pluripotency markers AP, SSEA-1, and OCT3/4 (data not shown). On the transcriptional level, as assessed by real-time PCR, the overexpression of Pramel7 induced a slight increase of most of the pluripotency genes (Fig. 1D). Accordingly, on Cre-recombination the expression of Nanog and OCT3/4 reverted to wt levels in combination with an increase in fibroblast growth factor 5 (FGF5) (Fig. 1D). Overexpression of Pramel6 did not induce significant changes on the expression of the investigated genes (Fig. 1F). Nanog overexpression did not influence the transcriptional levels of Pramel7, indicating that *Pramel7* is not a target of Nanog (Supporting Information Fig. 2D).

Overexpression of Pramel7 Liberates ESCs from LIF Dependence and Maintains Pluripotency

We further analyzed the ability of ESCs overexpressing either Pramel6 or Pramel7 to be propagated clonally on *Lif*-knockout feeders [16] without LIF in the medium. After 11 days, E14 ESCs exhibited widespread differentiation and no longer expressed the pluripotency markers OCT3/4, SSEA-1, and AP (Fig. 2A, 2B and Supporting Information Fig. 3A). In contrast, Pramel7-overexpressing cells (referred as Pramel7 cells) were undistinguishable from Nanog-overexpressing cells (Supporting Information Fig. 3A) and only less than 10% of

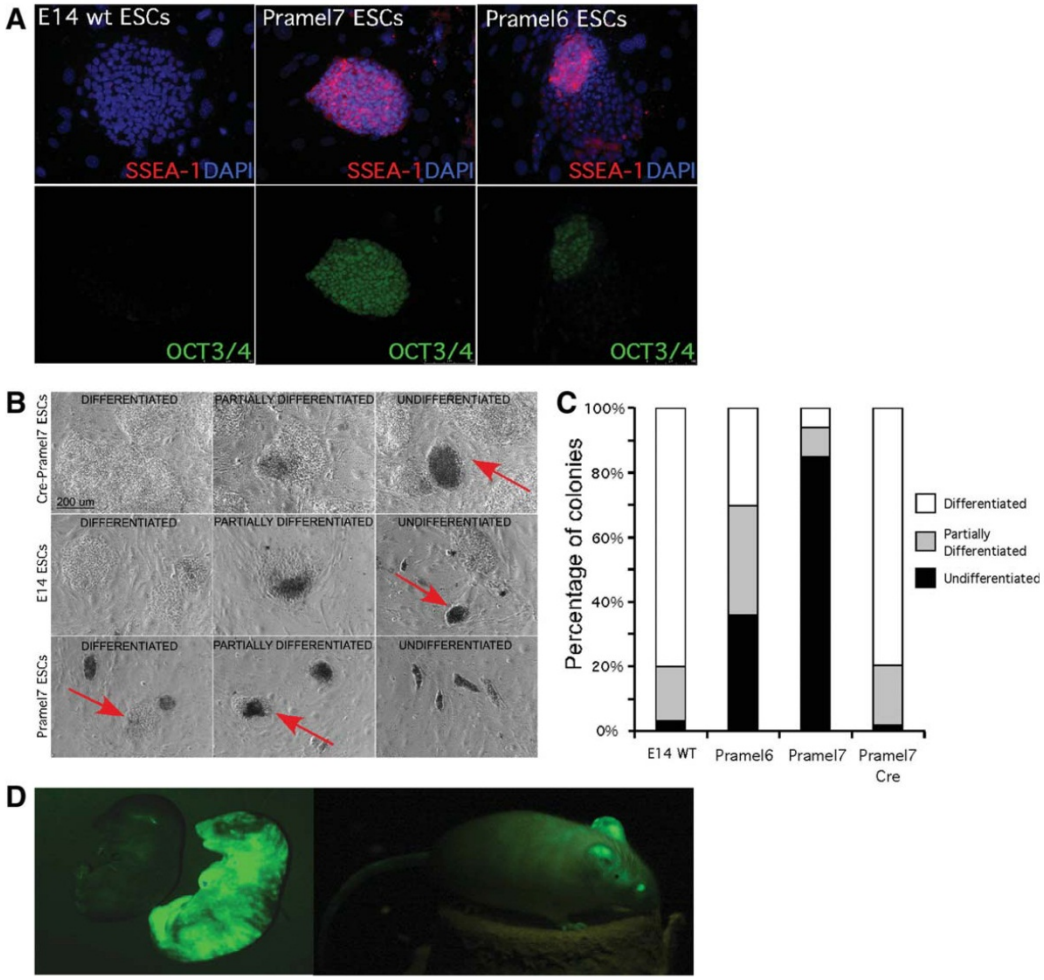


Figure 2. Characterization of Pramel7 and Pramel6 transgenic ESCs. **(A):** Immunostaining for OCT3/4 and SSEA-1 in E14 wt, Pramel6 and Pramel7 overexpressing ESCs cultivated for 11 days in absence of leukemia inhibitory factor (LIF) on a layer of *Lif* knockout feeders. Magnification: ×20. **(B, C):** Alkaline phosphatase (AP) staining and quantification of AP-positive colonies after 11 days in absence of LIF (100 colonies were counted). **(D):** Contribution of Pramel7 Cre-deleted cells to embryo and adult chimeric animals. Chimerism was assessed by green fluorescence. Wild-type pup (EGFP negative), EGFP-positive Cre-reverted Pramel7 pup and the adult chimera are shown. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; SSEA-1, stage-specific embryonic antigen-1.

the colonies showed no AP activity (Fig. 2C). Accordingly, Cre-recombined Pramel7 ESCs (referred as Cre-Pramel7 cells) behaved like wt ESCs and exhibited more than 80% of differentiated colonies (Fig. 2C). In contrast, only about one-third of the colonies in Pramel6-overexpressing clones remained completely undifferentiated (Fig. 2C and Supporting Information Fig. 3A). Our data reveals that overexpression of Pramel7, but not of Pramel6, is sufficient to liberate ESCs from LIF dependence and to promote self-renewal.

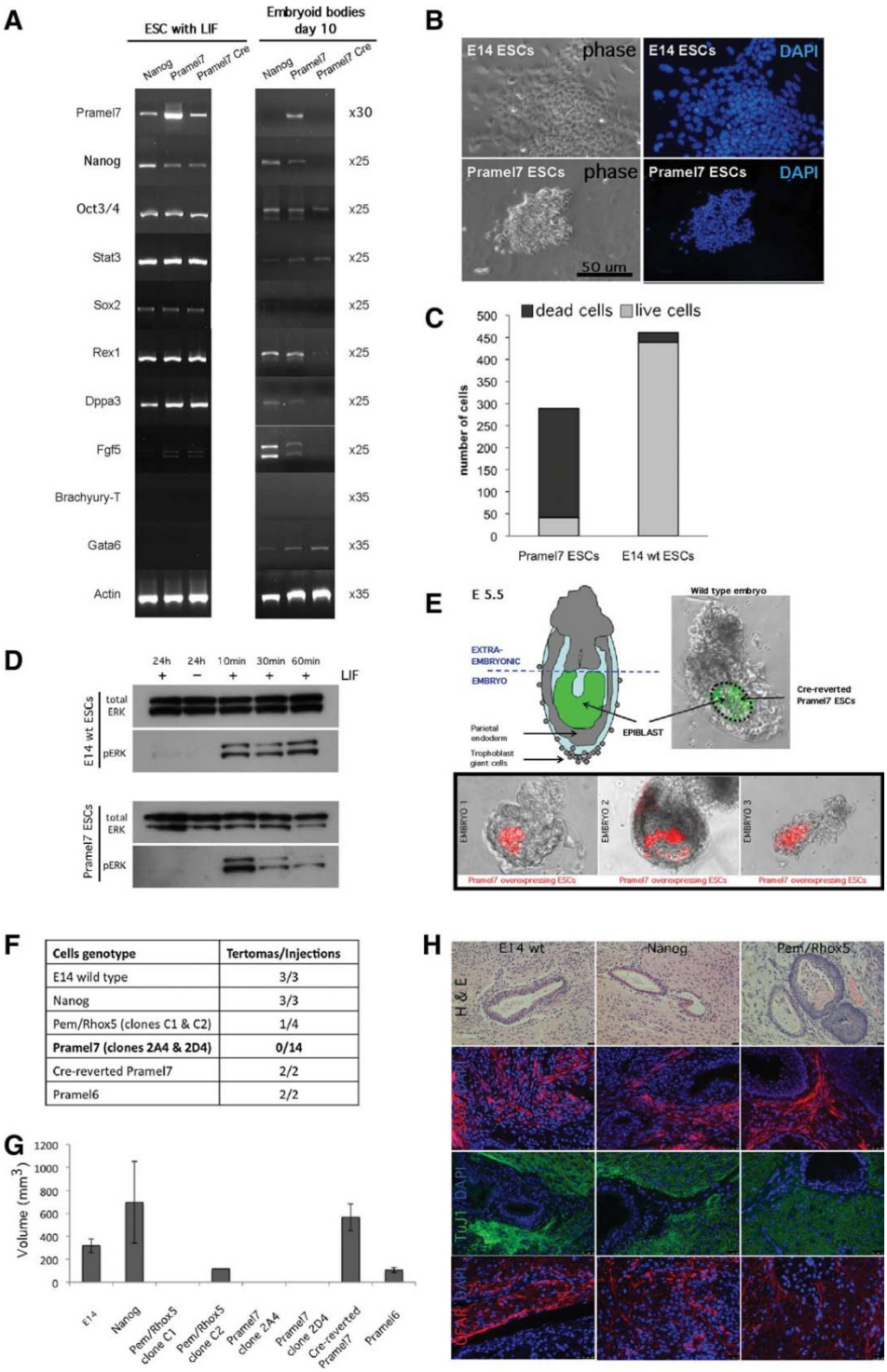
Finally, we investigated whether cells maintained in a self-renewing pluripotent state exclusively through overexpression of Pramel7 were retaining their embryo colonization capacity. Excision of *Pramel7* restored LIF dependence and these cells contributed to the generation of healthy live chimeras upon morula aggregation (Fig. 2D and Supporting Information Table 3).

Pramel7-Overexpressing ESCs Fail to Differentiate In Vitro

To investigate the role of Pramel7 in ESC differentiation, embryoid bodies were generated with Pramel7, Cre-Pramel7, and Nanog-overexpressing ESCs. As assessed by reverse transcription and PCR, expression of pluripotency markers, such as OCT3/4, Nanog, and REX1, was persisting after 10 days in Pramel7 cells as well as in the Nanog-overexpressing cells, whereas it was completely abolished in Cre-revertants (Fig. 3A). To further assess the capacity of Pramel7 cells to undergo defined differentiation, cells were cultivated with neural differentiation medium. E14 cells differentiated, whereas the majority of Pramel7 cells showed a large number of cells with pyknotic nuclei (Fig. 3B, 3C) indicating a high degree of cell death.

Pramel7 Maintains Pluripotency In Vitro Through Gradual Suppression of ERK Phosphorylation

ERK phosphorylation is known to be an early signaling event required for the differentiation of ESCs, and it has been proposed that the balance between LIF-induced activation of STAT3 and ERK may determine the efficiency of self-renewal and thereby influence stem cell fate [17, 18]. To further understand the mechanism underlying Pramel7-mediated



Cells genotype	Tertomas/Injections
E14 wild type	3/3
Nanog	3/3
Pem/Rhox5 (clones C1 & C2)	1/4
Pramel7 (clones 2A4 & 2D4)	**0/14**
Cre-reverted Pramel7	2/2
Pramel6	2/2

Figure 3. Pramel7 overexpression results in differentiation defects in vitro and in vivo. **(A):** Expression levels of pluripotency genes (Pramel7, Nanog, Oct3/4, Pem/Rhox5, Stat3, Sox2, Rex1, Dppa3), endoderm (Gata-6), primitive ectoderm (Fgf5), and mesoderm (Brachyury-T) were measured by semiquantitative RT-PCR in ESCs (control) and in embryoid bodies generated from Pramel7-overexpressing cells, Pramel7-Cre revertants, and Nanog-overexpressing cells. Number of PCR cycles is annotated. **(B, C):** In vitro neural differentiation of Pramel7-overexpressing cells and parental E14 cells. Pramel7-overexpressing clones exhibited an extensive number of cells with pyknotic nuclei, as shown by DAPI staining. Magnification: ×20. Number of live and dead cells after 4 days of neural differentiation: Cells which showed condensed nuclei were defined as dead cells, whereas normal nuclei were considered as live cells. **(D):** Western blotting for phospho-Erk after LIF stimulation of wt and Pramel7-overexpressing ESCs for 10, 30, or 60 minutes reveals that Pramel7 maintains pluripotency through gradual suppression of pERK. **(E):** Morula aggregation of Cre-reverted Pramel7 cells (EGFP positive) and Pramel7-overexpressing cells (RFP labeled). Pramel7-overexpressing cells do not correctly take part to the development, whereas Cre-reverted Pramel7 cells integrate into the developing embryo and at E5.5 they are part of the epiblast. **(F–H):** Teratoma formation reveals impaired in vivo differentiation potential of Pramel7-overexpressing cells. Efficiency of teratoma formation in E14 wt ESCs, Nanog, Pem/Rhox5, Pramel6, and Pramel7-overexpressing cells was assessed by number of teratomas generated **(F)** and tumor volume (mm^3) measurement **(G)**, as well as by degree of tumor differentiation **(H)** analyzed by immunostaining for SMA, TuJ1, and GFAP. Magnification: ×10. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acid protein; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblasts; pERK, phosphorylated ERK; SMA, smooth muscle actin; TuJ1, βIII-tubulin.

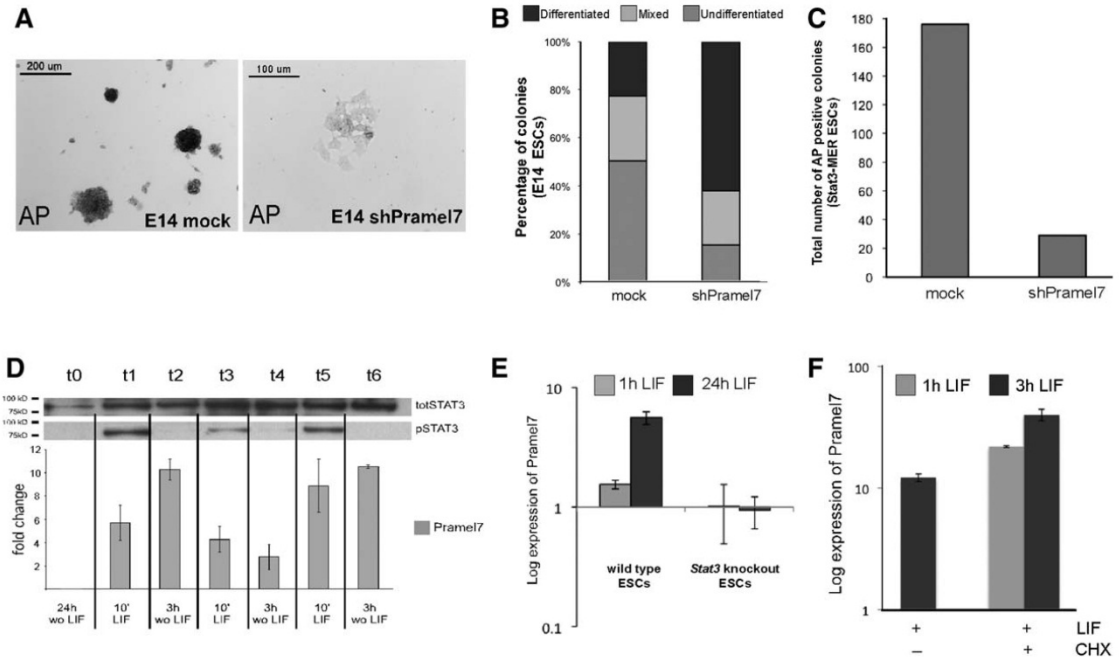


Figure 4. Pramel7 expression is indispensable for LIF/STAT3-dependent maintenance of pluripotency and is a direct STAT3 target gene. **(A, B):** Representative appearance of AP-stained colonies **(A)** after Pramel7 knockdown in E14 ESCs in presence of LIF. Control (E14 mock), Pramel7 knockdown (E14 shPramel7). Quantification of AP-positive colonies **(B)** after Pramel7 knockdown in E14 wt cells. Colonies that showed more than 80% positive cells were identified as "undifferentiated," 20%–80% as "mixed," and less than 20% as "differentiated". **(C):** Pramel7 knockdown in STAT3MER cells cultivated in presence of 4-hydroxy-tamoxifen. Total number of AP-positive colonies is presented. **(D):** Time-pulse assay with LIF in E14 wt ESCs. Phosphorylation levels of STAT3 (pSTAT3) were monitored by Western blot, whereas the amount of Pramel7 mRNA was measured by real time-polymerase chain reaction (PCR). **(E):** Real-time PCR analysis of Pramel7 mRNA expression in wt and *Stat3* null ESCs cells after LIF stimulation for 1 or 24 hours. **(F):** STAT3 directly transcribes *Pramel7* gene. Real-time PCR analysis of Pramel7 mRNA expression in E14 wt ESCs deprived from LIF for 24 hours followed by LIF stimulation for 1 or 3 hours in the presence or absence of cycloheximide (50 μg/ml). Abbreviations: AP, alkaline phosphatase; LIF, leukemia inhibitory factor.

self-renewal, we investigated by Western blotting activation of ERK in E14, Pramel6, Pramel7, and in Cre-recombined ESCs on LIF stimulation. Twenty-four hours LIF-depleted wt, Pramel6-, and Cre-Pramel7 cells showed progressive phosphorylation of ERK (pERK) on LIF induction (Supporting Information Fig. 3B). In Pramel7 cells, however, pERK was only detectable 10 minutes after LIF stimulation and drastically decreased after 30 minutes (Fig. 3D). Taken together, this data suggests that Pramel7 overexpression prevents ESCs from differentiation by promoting ERK dephosphorylation.

Pramel7 Cells Are Unable to Form Teratomas and to Contribute to Embryo Development

To test the capacity of Pramel7 cells to contribute to embryo development, we genetically labeled these cells with red fluorescent protein (RFP) and performed morula aggregations. After being transferred into the uterus of foster mothers, at E5.5 the embryos were isolated and analyzed. Embryos aggregated with Pramel7-RFP cells exhibited malformations (Fig. 3E and Supporting Information Table 3), indicating that Pramel7 cells do not enter normal development.

We further investigated the ability of Pramel6 and Pramel7 cells to form teratomas. We transplanted 10^6 ESCs subcutaneously into immunoincompetent nonobese diabetic/severe combined immunodeficiency mice (Fig. 3F). After 3 weeks, wt and Cre-Pramel7 ESCs produced teratomas of similar size (Fig. 3G and Supporting Information Fig. 4A) containing derivatives of all three germ layers (Supporting Information Fig. 4B). Pramel6 cells generated similar teratomas but with smaller volume than the ones generated by the control clones (Fig. 3G and Supporting Information Fig. 4A, 4B). Intriguingly, 14 independent injections with two different Pramel7 ESC-clones overexpressing similar amounts of Pramel7 never generated teratomas, even after 2 months incubation. Seeing that no previous publication describes a similar behavior of pluripotent cells, we tested the potential of teratoma formation of ESCs overexpressing Nanog or PEM/RHOX5. A total of 100% of the injections with Nanog-overexpressing ESCs produced teratomas undistinguishable in size (Fig. 3G) and histological composition from the ones generated from wt and Cre-revertant cells (Fig. 3H, Supporting Information Fig. 4A and 4B). This data indicates that Nanog overexpression is not sufficient to prevent differentiation in the context of teratoma.

ESCs-overexpressing PEM/RHOX5 were previously shown to form teratomas containing undifferentiated cells [13]. Interestingly, 75% of the injections with PEM/RHOX5-overexpressing cells failed to generate teratomas, in a similar way to the Pramel7 cells (Fig. 3G). Nevertheless, the only teratoma isolated contained differentiated tissue similar to the wt and Nanog cells (Fig. 3H). In summary, overexpression of Pramel7 impairs the capacity of ESCs to generate teratomas and the inability to differentiate probably causes the death of the cells.

Pramel7 Is Necessary for LIF- and STAT3-Dependent Maintenance of Pluripotency in ESCs

To investigate whether Pramel7 is required for maintenance of pluripotency in ESCs, we performed knockdown experiments in E14 wt ESCs cultivated in the presence of LIF.

Knockdown was achieved by transient ESCs transfection with a cocktail of four shRNA-containing vectors specific for Pramel7. Four days after transfection, Pramel7 mRNA was completely silenced, although the efficiency decreased 4–5 times in comparison with the control cells at day 6 (data not shown). Despite the presence of LIF, knockdown of Pramel7 induced loss of AP activity and differentiation (Fig. 4A, 4B), whereas control cells did not. The data indicates that LIF-mediated self-renewal in ESCs depends on Pramel7 expression.

To further assess whether self-renewal induced by STAT3 overexpression also depends on Pramel7, we exploited STAT3MER ESCs, which conditionally express a STAT3 fusion protein with a mutated estrogen receptor. These cells maintain pluripotency in the absence of LIF by the sole activation of STAT3MER with 4-OHT [19] and were shown to upregulate Pramel7 [14]. Surprisingly, STAT3MER activation failed to promote stem cell maintenance in Pramel7 knockdown cells, resulting in a drastic decrease in the total number of AP-expressing colonies (Fig. 4C). These results clearly indicate that the expression of Pramel7 is essential for both LIF- and STAT3-mediated maintenance of pluripotency.

Pramel7 Is a Novel Direct Downstream Target of STAT3 in the LIF/STAT3 Pathway

On the basis of our previous [14] and recent results, we assessed whether Pramel7 might represent a novel effector of the LIF/STAT3-pathway. We therefore first monitored STAT3 phosphorylation and Pramel7 mRNA expression in response to three cycles of 10 minutes of LIF incubation. Depletion of LIF for 24 hours (t0) and successive incubation with LIF-containing medium for 10 minutes (t1) induced in E14 cells a fast phosphorylation of STAT3 (pSTAT3) as assessed by Western blotting (Fig. 4D). Concomitantly, mRNA levels of Pramel7 strongly increased during this period (Fig. 4D). The same behavior was observed also after the third round of LIF incubation, suggesting that *Pramel7* is a downstream target of LIF/STAT3.

To test the robustness of this hypothesis, we monitored Pramel7 expression after LIF induction in *Stat3* null cells [12] cultivated in N2B27 + 2i medium without LIF. In the wt cells, LIF induced an upregulation of Pramel7 mRNA, whereas *Stat3* knockout cells failed to regulate Pramel7 expression (Fig. 4E). These results clearly indicate that *Pramel7* transcription is STAT3-dependent. Furthermore, LIF stimulation in wt ESCs, in the presence of the protein synthesis inhibitor cycloheximide, resulted in strong activation of *Pramel7* transcription (Fig. 4F) confirming that STAT3 directly drives *Pramel7* transcription.

Pramel7 Expression Is Regulated Through the Parallel Circuitry of the LIF Signal Pathways

We observed that within 10 minutes LIF induces a rapid upregulation of Pramel7 transcripts under standard culture conditions (Fig. 4D). Interestingly, the presence of 2i causes a significant delay in the LIF-dependent Pramel7 upregulation (Fig. 4E). In the presence of 2i, LIF supply is dispensable and the cells can be propagated in the absence of active LIF/STAT3 pathway [15]. We therefore considered whether the retarded responsiveness of *Pramel7* transcription to LIF was due to the absence of active STAT3 or to the presence of the ERK- and/or GSK3β-inhibitors.

We examined induction of Pramel7 on addition of LIF in absence of 2i or in presence of either PD0325901 (ERK-inhibitor) or CHIR99021 (GSK3β-inhibitor). ESCs were then incubated for 30 minutes, 1 hour, 3 hours, or 5 hours with LIF. Intriguingly, the presence of CHIR99021 completely blocked *Pramel7* transcription (Fig. 5A) indicating that the inhibition of GSK3β activity impairs STAT3-mediated transcription of *Pramel7*. We therefore analyzed the effect of LY294002, a phosphoinositide-3-kinase (PI3K) inhibitor on *Pramel7* regulation. Because of the known apoptotic effect of PI3K inhibition, cells were incubated with low inhibitor concentrations and for a short time. STAT3 mRNA expression was not influenced by the presence of the PI3K inhibitor and as expected, increased during the LIF incubation times. Whereas the level of Pramel7 transcripts remained unaltered even after 5 hours of LIF stimulation (Fig. 5B). We can therefore conclude that *Pramel7* transcription is mediated by the parallel activity of the LIF/STAT3 and the PI3K/GSK3β pathways.

It is known that the sole inhibition of ERK or GSK3β is not sufficient for maintaining ESCs undifferentiated. To test whether Pramel7 overexpression can overcome this situation, we cultivated wt and Pramel7 cells in N2B27 medium supplemented only with CHIR99021. After 5 days, wt cells showed significant upregulation of the differentiation markers Brachyury-T, GATA6, and GATA4, and after 9 days, the expression of REX1, Nanog, OCT3/4, and SOX2 was reduced (Fig. 5C). Immunohistochemistry for OCT3/4 and SSEA-1 showed a reduced expression in wt cells and colonies started to spread out loosing their compact shape (Fig. 5D). In contrast, Pramel7 cells formed high compact colonies, which were homogeneously positive for both pluripotency markers (Fig. 5D). Moreover, they formed more than 70% undifferentiated, homogenously AP-positive colonies, whereas E14 cells were only about 25% (Fig. 5E). This demonstrates that overexpression of Pramel7 in combination with the GSK3β-inhibitor is sufficient to maintain ESCs undifferentiated, indicating that the presence of Pramel7 can compensate for the need of MEK/ERK inhibition.

Pramel7 Overexpression Under Feeder- and Serum-Free Conditions Is Only Partially Able to Maintain Pluripotency

We asked next if Pramel7 is able to maintain ESCs undifferentiated in N2B27 medium in the absence of feeders. E14 and Pramel7 ESCs were adapted to the N2B27 + 2i conditions (without LIF) by extensive passaging and both cell lines after 11 passages showed homogenous AP staining (Fig. 6A). We therefore used these cells for our further experiments. We first investigated if overexpression of Pramel7 is sufficient for prolonged cell expansion in N2B27 medium without 2i and LIF, a condition that normally does not support self-renewal. After two passages at clonal density, wt cells died, whereas Pramel7 cells could be split for one more passage, but finally they also died (Fig. 6B). This indicates that the sole overexpression of Pramel7 is not sufficient for prolonged cells expansion under these conditions. We therefore investigated if Pramel7 cells were able to self-renew in N2B27 medium supplemented with LIF alone, a condition that is also normally not sufficient for maintaining ESCs. After passaging, almost all the E14 cells either died or stopped to self-renew. However, Pramel7 cells showed higher self-renewing rate and formed round, compact colonies (Fig. 6C). OCT3/4 and SSEA-1 expression was detected only in the transgenic cells, but not in the wt E14 cells (Fig. 6D). Quantification for AP-positive colonies showed that Pramel7 cells exhibited 5% of completely undifferentiated colonies and about 50% of partially differentiated colonies when stained for AP (Fig. 6E). In contrast, more than 80% of the wt cells were completely differentiated and showed no AP activity at all (Fig. 6E). This was also

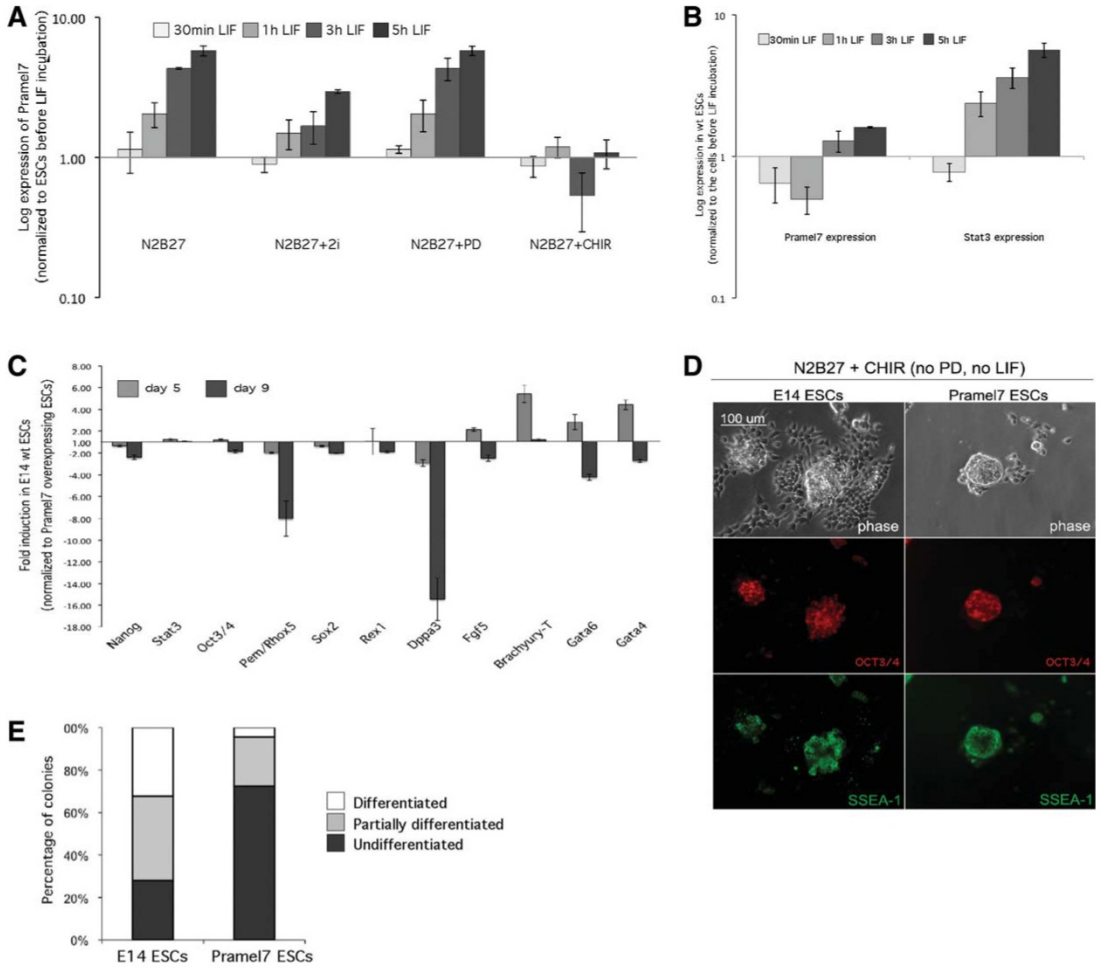


Figure 5. Transcriptional regulation of Pramel7. **(A):** Quantitative real-time polymerase chain reaction (PCR) analysis of Pramel7 gene expression in E14 wild-type (wt) ESCs stimulated with LIF in presence of 2i, PD0325901 (PD), or CHIR99021 (CHIR) at different time points. **(B):** Quantitative real-time PCR analysis of Pramel7 and Stat3 expression in E14 wt ESCs stimulated with LIF in presence of LY294002. **(C):** Gene expression analyses in E14 wt cells cultivated for 5 or 9 days in the presence of CHIR99021. **(D):** Immunostaining for SSEA-1 and OCT3/4 of E14 and Pramel7 cells after 5 days in N2B27 medium supplemented only with CHIR99021. Transgenic Pramel7 cells retained the expression of OCT3/4 and SSEA-1 markers, whereas E14 wt cells differentiated. **(E):** Quantification of alkaline phosphatase-positive colonies after 9 days of culture in N2B27 medium supplemented only with CHIR99021. Abbreviation: LIF, leukemia inhibitory factor.

confirmed by real-time PCR (Fig. 6F). In summary, the combination of Pramel7 overexpression and LIF increases self-renewal capacity of ESCs, facilitating the maintenance of the undifferentiated state. Nevertheless, this synergistic effect was only partial, as complete elimination of differentiation was not observed.

Discussion

The JAK/STAT3-pathway was shown to be essential and sufficient in mouse ESCs to mediate LIF signals thereby contributing to the maintenance of pluripotency [19, 20]. Even though a complete bypass of LIF signaling is possible under certain circumstances [15], optimal self-renewal is obtained by combination of LIF and 2i, confirming LIF/STAT3-pathway as an essential component in ESCs. Moreover, STAT3-activation was recently described to be a limiting factor for reprogramming to ground state pluripotency [21]. Expression of Pramel6 and Pramel7 was increased upon overexpression of STAT3 in ESCs [14] and therefore represent potential candidates involved in regulating the homeostasis of ESCs [14, 22, 23].

Here, we demonstrate that *Pramel7* is not only a direct target of STAT3 but also that STAT3-mediated maintenance of pluripotency strongly depends on Pramel7 expression. During the preimplantation embryo stages the LIF/gp130 pathway is dispensable for early development without diapause [24]. Nevertheless, even in the absence of LIF stimuli, Pramel7 is expressed in the inner part of the morula and in the ICM of the embryo. Intriguingly, in *Stat3*-knockout cells, where the LIF/STAT3-pathway is not active, Pramel7 expression was upregulated compared with the wt cells. These findings suggest that there might be LIF/STAT3 compensatory mechanisms or factors, which drive and regulate Pramel7 expression. We exclude the possibility of an autoregulatory activity of Pramel7, as the protein lacks the characteristic domains

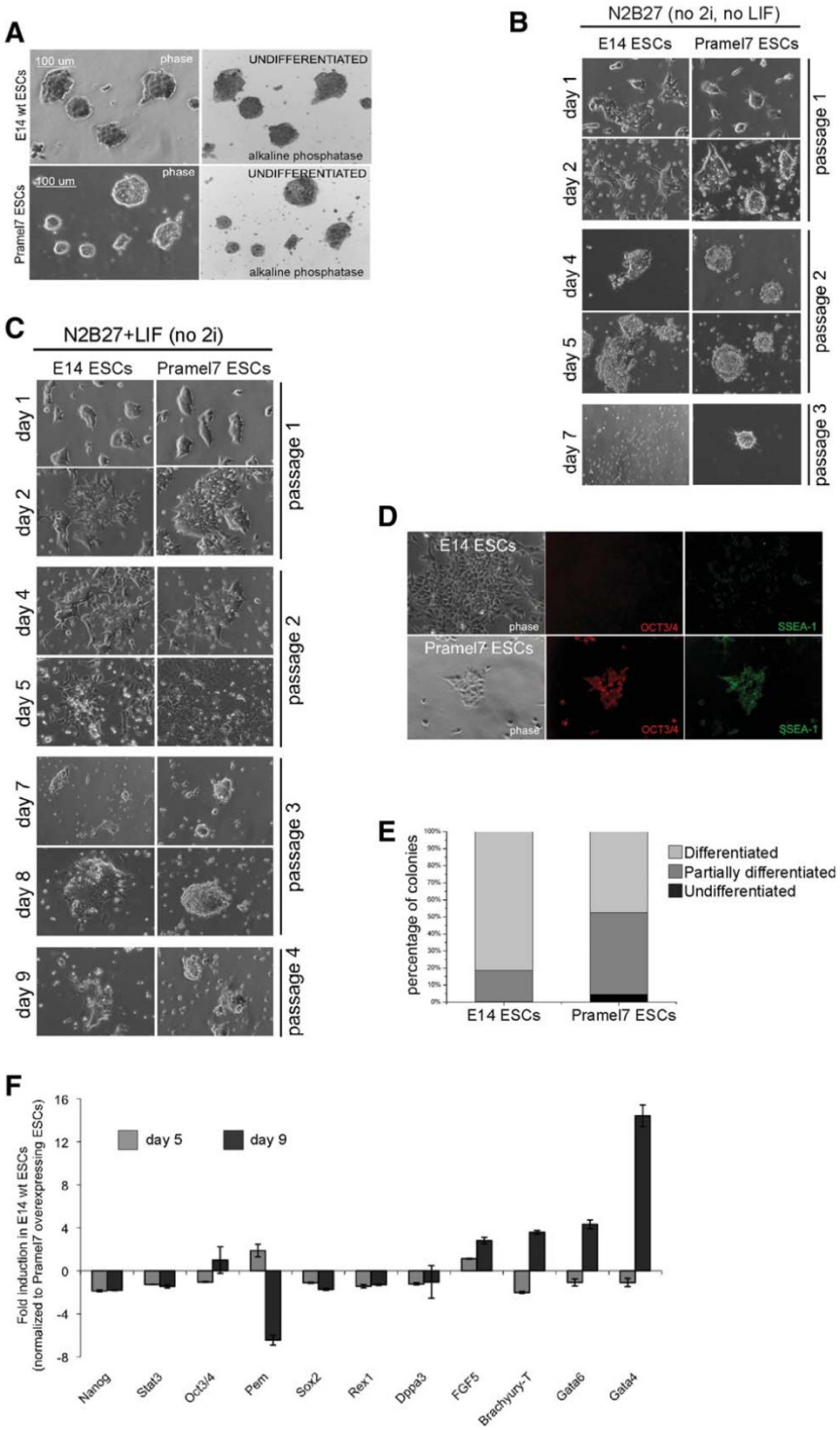


Figure 6. The sole Pramel7 overexpression in serum- and feeder-free condition is not completely sufficient for maintaining the undifferentiated state of ESCs. **(A):** Representative appearance of alkaline phosphatase (AP)-positive colonies in E14 wt and Pramel7-overexpressing cells after 11 passages in N2B27 + 2i medium (feeders free and LIF free). Both cell lines show AP activity. **(B):** Representative morphology of wt and Pramel7 ESCs after three passages in N2B27 medium in absence of 2i and LIF. **(C):** E14 and Pramel7 cells cultivated for four passages in N2B27 medium supplemented with LIF only. **(D):** Immunostaining for OCT3/4 and SSEA-1 expression in wt and Pramel7-overexpressing cells cultivated in N2B27+LIF. E14 cells lost OCT3/4 and SSEA-1 expression, whereas Pramel7 cells remained undifferentiated. **(E):** Quantification of AP-positive colonies after 9 days of culture in N2B27 medium supplemented only with LIF. **(F):** Gene expression analysis of ESCs cultivated 5 and 9 days in N2B27+LIF. E14 cells showed progressively downregulation of pluripotency genes and upregulation of differentiation marker genes. Abbreviations: LIF, leukemia inhibitory factor; SSEA-1, stage-specific embryonic antigen-1.

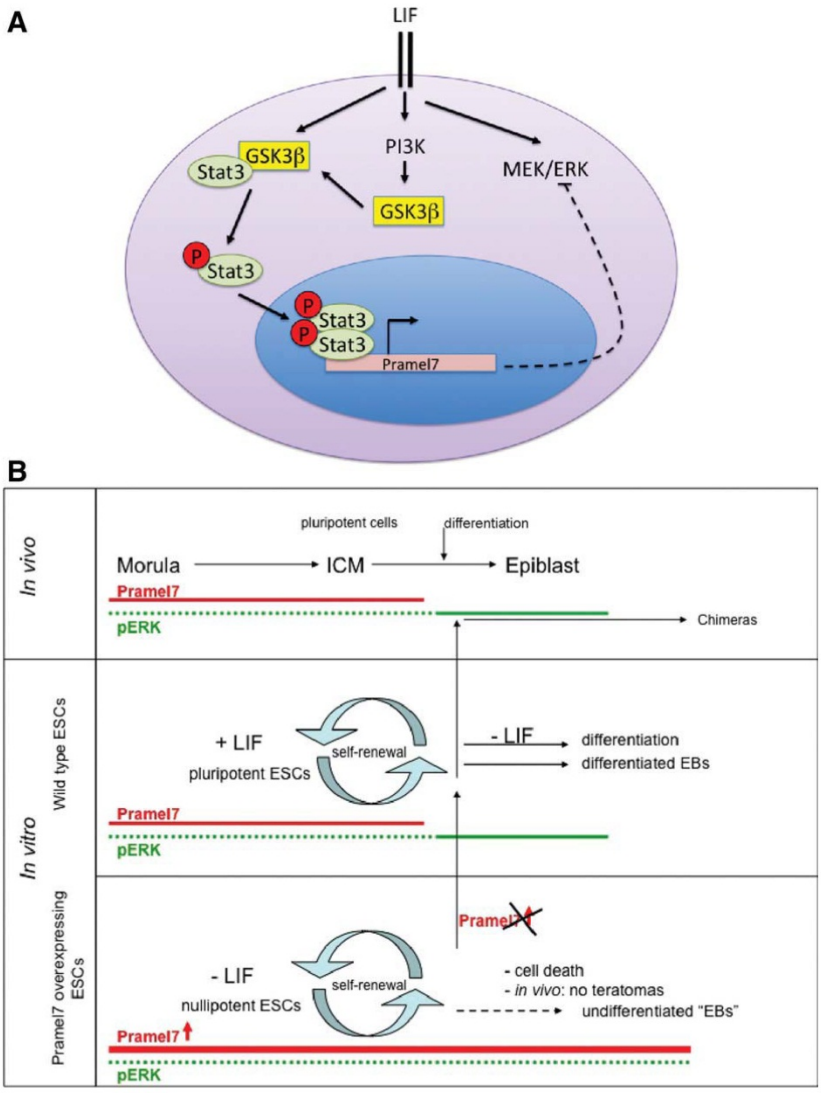


Figure 7. Hypothetical transcriptional mechanisms, which drive Pramel7 expression. **(A):** Schematic representation of *Pramel7* transcription directly controlled by the LIF/STAT3-pathway and also by the LIF/PI3K-pathway. LIF/gp130 receptor leads to the activation of three different pathways, that is, the LIF/STAT3, the LIF/PI3K/GSKβ3, and the LIF/MEK/ERK-pathway. *Pramel7* transcription is directly controlled by the transcription factor STAT3, whereas its phosphorylation is probably regulated by the GSK3β kinase. The combinatorial effect of LIF/STAT3 and GSK3β drives and controls *Pramel7* transcription, which in turn blocks phosphorylation of ERK and therefore ESCs differentiation. **(B):** Schematic representation of Pramel7 expression and effects in vivo and in vitro. In the late preimplantation embryo, Pramel7 expression is only detectable in the inner part of the morula and in the ICM of the blastocyst. Immediately after implantation Pramel7 expression disappears, when differentiation processes occur in the embryo. In vitro wild-type (wt) ESCs do express Pramel7 but need LIF for self-renewing. After LIF depletion wt ESCs differentiate into different cell types and also form EBs. In Pramel7-overexpressing cells, addition of LIF in the medium is not necessary anymore for maintaining the undifferentiated and self-renewing state of the cells. These cells are not able to differentiate and either form undifferentiated embryoid bodies or die. Once the overexpression of Pramel7 is reverted to the wt level, these cells are able to differentiate and take part to embryo development. Abbreviations: EBs, embryoid bodies; ERK, extracellular signal-regulated kinase; ICM, inner cell mass; LIF, leukemia inhibitory factor; MEK, MAPK/ERK Kinase; pERK, phosphorylated ERK.

typical for transcriptional factors but contains LRR domains mediating protein-protein interactions [25].

Pramel7 regulation occurs through a parallel circuit involving both the STAT3 and the PI3K-pathway. The regulation of GSK3β by PI3K is involved in the transcription of *Pramel7* upon LIF stimulation and thus for the maintenance of LIF-mediated control of ESC self-renewal. We suggest that GSK3β is directly involved in the control of STAT3-mediated *Pramel7*-transcription, but the exact nature of this regulation remains to be elucidated. Interestingly, recent data suggests that STAT3-activation is dependent on GSK3β [26]. Beurel et al. reported that GSK3β does not function upstream of STAT3-activating tyrosine kinases but instead is required for the recruitment of STAT3 to the receptor and for its tyrosine phosphorylation-mediated activation. Even though the authors did not directly analyze ESCs, they demonstrated that the dependence of STAT3-activation on GSK3β was a widespread regulatory interaction observed with multiple stimuli and in several types

of cells [26], suggesting that the same molecular process likely occurs in ESCs. This is interesting because it was previously shown that in ESCs, STAT3 is not a target of PI3K action and that the loss of self-renewal and the consequent differentiation of the cells after inhibition of PI3K was due to an increase in pERK upon LIF-stimulation [27]. The ERK-pathway is continuously activated in undifferentiated ESCs predominantly by signaling through FGF receptor [28]. It is widely accepted that in self-renewing ESC cultures, the provision of LIF and the following activation of STAT3 acts downstream of pERK to override the auto inductive capacity of FGF4. In this study, we identify for the first time Pramel7 as a protein that links the three LIF/gp130-induced pathways (LIF/STAT3, LIF/MEK/ERK, and LIF/PI3K/GSK3β). We suggest that the concerted activity of STAT3 and GSK3β controls *Pramel7* transcription, which in turn regulates the phosphorylation of ERK leading to an abrogation of ESC differentiation (Fig. 7A).

Importantly, Pramel7 blocks teratoma formation capacity and differentiation potential in vitro and in vivo, indicating that *Pramel7* silencing is an absolute requirement for differentiation (Fig. 7B). Similar to Pramel7 overexpression, forced expression of Nanog confers LIF-independent self-renewal and prevents differentiation of ESCs [11]. Unlike Pramel7-overexpressing cells, though, Nanog-overexpressing clones were able to generate teratomas-containing derivatives of all three germ layers. Even though forced expression of both genes in absence of LIF leads to self-renewal of ESCs, our data highlights a different reaction to differentiation stimuli and therefore a different function of these genes in maintaining pluripotency. Nanog is expressed at similar levels in both ICM and ESCs, whereas Pramel7 expression is higher in the ICM than in ESCs [22]. Moreover, it was reported that the essential function of Nanog is to define the pluripotent pool of cells of the ICM and the germ cells, and once the cells are established, its function is dispensable, so that *Nanog* null ESCs are able to self-renew [29]. Our data also confirms the idea that Nanog is important for the pluripotent identity of the ICM and of ESCs, whereas Pramel7 is more probably involved in allowing/blocking the start of differentiation rather than actively taking part in the processes maintaining pluripotency. In agreement with this hypothesis, elimination of Pramel7 expression by knockdown induces ESC differentiation independently if LIF or even STAT3 overexpression is present. Taken together, this suggests that Nanog is priming a cell to become pluripotent, whereas Pramel7 inhibits a pluripotent ESC from commitment and suggests that Pramel7 acts as the judge in the trial between pluripotency and differentiation. Its presence maintains the cells in a self-renewing state by retarding commitment.

Assuming that Pramel7 is not directly acting as a transcriptional factor but solely through binding to other proteins, further experiments aimed at the identification of potential Pramel7 binding partners are necessary to reveal the mechanisms underlying maintenance of pluripotency through Pramel7.

Conclusion

Our data show that the combined activity of STAT3 and GSK3 controls *Pramel7* transcription, which in turn regulates the phosphorylation of ERK leading to the inhibition of ESC differentiation. Accordingly, Pramel7 ablation causes ESC differentiation, whereas its overexpression sustains long-term self-renewal in the absence of LIF. These observations prove Pramel7 as an essential factor of the signaling network regulating pluripotency and self-renewal in ESCs.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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